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(71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).

(72) Inventors: KNUDSEN, Liselotte, Bjerre; Valby Langgade 49A, 1. tv., DK-2500 Valby (DK). HUUSFELDT, Per, Olaf; Applebys Plads 27, 5. mf., DK-1411 Copenhagen K (DK). NIELSEN, Per, Franklin; Dalsø Park 59, DK-3500 Værløse (DK). KAARSHOLM, Niels, C.; Clausholmvej 38, DK-2720 Vanløse (DK). OLSEN, Helle, Birk; Skolelodden 23, DK-3450 Allerød (DK). BJØRN, Søren, Erik; Marie Grubbes Allé 47, DK-2800 Lyngby (DK).

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(57) Abstract

The present invention relates to a pharmaceutical composition comprising a GLP-1 derivative of improved solubility and/or stability, and to a method for improving the solubility and/or stability of GLP-1 or a fragment or an analogue thereof.

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GLP-1 DERIVATIVES WITH HELIX-CONTENT EXCEEDING 25 %, FORMING PARTIALLY STRUCTURED MICELLAR-LIKE AGGREGATES

Field of the invention

The present invention relates to a pharmaceutical composition comprising a GLP-1 derivative of improved solubility and/or stability, and to a method for improving the solubility and/or stability of GLP-1 or a fragment or an analogue thereof.

Background of the invention

Peptides are widely used in medical practice, and since they can be produced by recombinant DNA technology it can be expected that their importance will increase also in the years to come.

The hormones regulating insulin secretion belong to the so-called enteroinsular axis, designating a group of hormones, released from the gastrointestinal mucosa in response to the presence and absorption of nutrients in the gut, which promote an early and potentiated release of insulin. The enhancing effect on insulin secretion, the so-called incretin effect, is probably essential for a normal glucose tolerance. Many of the gastrointestinal hormones, including gastrin and secretin (cholecystokinin is not insulinotropic in man), are insulinotropic, but the only physiologically important ones, those that are responsible for the incretin effect, are the glucose-dependent insulinotropic polypeptide, GIP, and glucagon-like peptide-1 (GLP-1). Because of its insulinotropic effect, GIP, isolated in 1973 (1) immediately attracted considerable interest among diabetologists. However, numerous investigations carried out during the following years clearly indicated that a defective secretion of GIP was not involved in the pathogenesis of insulin dependent diabetes mellitus (IDDM) or non insulin-dependent diabetes mellitus (NIDDM) (2). Furthermore, as an insulinotropic hormone, GIP was found to be almost ineffective in NIDDM (2). The other incretin hormone, GLP-1 is the most potent insulinotropic substance known (3). Unlike GIP, it is surprisingly effective in stimulating insulin secretion in NIDDM patients. In addition, and in contrast to the other insulinotropic hormones (perhaps with the exception of secretin) it also potently inhibits glucagon secretion. Because of these actions it has pronounced blood glucose lowering effects particularly in patients with NIDDM.

GLP-1, a product of the proglucagon (4), is one of the youngest members of the secretin-VIP family of peptides, but is already established as an important gut hormone with regulatory function in glucose metabolism and gastrointestinal secretion and metabolism (5). The glu-

cagon gene is processed differently in the pancreas and in the intestine. In the pancreas (9), the processing leads to the formation and parallel secretion of 1) glucagon itself, occupying positions 33-61 of proglucagon (PG); 2) an N-terminal peptide of 30 amino acids (PG (1-30)) often called glicentin-related pancreatic peptide, GRPP (10, 11); 3) a hexapeptide corresponding to PG (64-69); 4) and, finally, the so-called major proglucagon fragment (PG (72-158)), in which the two glucagon-like sequences are buried (9). Glucagon seems to be the only biologically active product. In contrast, in the intestinal mucosa, it is glucagon that is buried in a larger molecule, while the two glucagon-like peptides are formed separately (8). The following products are formed and secreted in parallel: 1) glicentin, corresponding to PG (1-69), with the glucagon sequence occupying residues Nos. 33-61 (12); 2) GLP-1(7-36)amide (PG (78-107))amide (13), not as originally believed PG (72-107)amide or 108, which is inactive). Small amounts of C-terminally glycine-extended but equally bioactive GLP-1(7-37), (PG (78-108)) are also formed (14); 3) intervening peptide-2 (PG (111-122)amide) (15); and 4) GLP-2 (PG (126-158)) (15, 16). A fraction of glicentin is cleaved further into GRPP (PG (1-30)) and oxyntomodulin (PG (33-69)) (17, 18). Of these peptides, GLP-1, has the most conspicuous biological activities.

The amino acid sequence of GLP-1 is given *i.a.* by Schmidt *et al.* (*Diabetologia* **28** 704-707 (1985). Although the interesting pharmacological properties of GLP-1(7-37) and analogues thereof have attracted much attention in recent years only little is known about the structure of these molecules. The secondary structure of GLP-1 in micelles has been described by Thorton *et al.* (*Biochemistry* **33** 3532-3539 (1994)), but in normal solution, GLP-1 is considered a very flexible molecule. Surprisingly, we found that derivatisation of this relatively small and very flexible molecule resulted in compounds whose plasma profile were highly protracted and still had retained activity (PCT application No. DK97/00340).

While much attention has been focused on the pharmacological properties of acylated GLP-1 derivatives, hitherto little is known about their physico-chemical and solution structural properties. Such knowledge is a prerequisite for rational handling during e.g. production, purification and formulation work and is eventually important for understanding of the structural basis for the protraction mechanism.

GLP-1 and analogues of GLP-1 and fragments thereof are potentially useful *i.a.* in the treatment of type 1 and type 2 diabetes. However, solubility limitations and the low stability against the actions of endogenous diaminopeptidyl peptidase limits the usefulness of these compounds, and thus there still is a need for improvements in this field. Accordingly, it is one object

of the present invention to provide pharmaceutical solutions comprising GLP-1 derivatives with improved solubility and stability.

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Summary of the invention

Human GLP-1 is a 37 amino acid residue peptide originating from preproglucagon which is synthesised *i.a.* in the L-cells in the distal ileum, in the pancreas and in the brain. Processing of preproglucagon to give GLP-1(7-36)amide, GLP-1(7-37) and GLP-2 occurs mainly in the L-cells. A simple system is used to describe fragments and analogues of this peptide. Thus, for example, Gly^{8}-GLP-1(7-37) designates a fragment of GLP-1 formally derived from GLP-1 by deleting the amino acid residues Nos. 1 to 6 and substituting the naturally occurring amino acid residue in position 8 (Ala) by Gly. Similarly, $Lys^{34}(N^{\varepsilon}$-tetradecanoyl)-GLP-1(7-37) designates GLP-1(7-37) wherein the ε-amino group of the Lys residue in position 34 has been tetradecanoylated. Where reference in this text is made to C-terminally extended GLP-1 analogues, the amino acid residue in position 38 is Arg unless otherwise indicated, the optional amino acid residue in position 39 is also Arg unless otherwise indicated and the optional amino acid residue in position 40 is Asp unless otherwise indicated. Also, if a C-terminally extended analogue extends to position 41, 42, 43, 44 or 45, the amino acid sequence of this extension is as in the corresponding sequence in human preproglucagon unless otherwise indicated.

PCT application No. DK97/00340 describes various GLP-1 derivatives that are found to be very protracted. Whereas GLP-1 and GLP-1 analogues are molecules to which no defined solution structure can be ascribed, we found that some of these protracted GLP-1 derivatives

may exist in a partially structured micellar-like aggregated form which is stable over a wide concentration range.

Circular Dichroism (CD) can be used to show that the GLP-1 derivatives have a certain partially structured conformation independent of their concentration. In contrast, for normal GLP-1(7-37) an increase in the helix content is seen with increasing concentration, from 10-15% to 30-35% (at 500 μM concentration) in parallel with peptide self-association. For the GLP-1 derivatives forming partially structured micellar-like aggregates in aqueous solution the helix content remains constant above 30% at concentrations of 10 μM. The aggregated structured conformation is an inherent property of the derivative present in water or dilute aqueous buffer without the need for any additional structure-inducing components.

Thus, in its broadest aspect, the present invention relates to a pharmaceutical composition comprising a GLP-1 derivative which has a helix content as measured by CD at 222 nm in H_2O at 22 ± 2 °C exceeding 25%, preferably in the range of 25% to 50%, at a peptide concentration of about 10 μM.

The size of the partially helical, micelle-like aggregates may be estimated by size-exclusion chromatography. Similarly, the apparent (critical micelle concentrations) CMC's of the peptides may be estimated from the concentration dependent fluorescence in the presence of appropriate dyes (e.g. Brito, R. & Vaz, W. (1986) Anal. Biochem. **152**, 250-255).

That the derivatives have a partially structured micellar-like aggregate conformation in aqueous solutions makes them more soluble and stable in solution as compared to the native peptide. The increased solubility and stability can be seen by comparing the solubility after 9 days of standing for a derivative and normal GLP-1(7-37) in a pharmaceutical formulation, e.g. 5 mM phosphate buffer, pH 6.9 added 0.1 M NaCl.

In the present text, the designation "an analogue" is used to designate a peptide wherein one or more amino acid residues of the parent peptide have been substituted by another amino acid residue and/or wherein one or more amino acid residues of the parent peptide have been deleted and/or wherein one or more amino acid residues have been added to the parent peptide. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent peptide or both.

The term “derivative” is used in the present text to designate a peptide in which one or more of the amino acid residues of the parent peptide have been chemically modified, *e.g.* by alkylation, acylation, ester formation or amide formation.

The term “a GLP-1 derivative” is used in the present text to designate a derivative of GLP-1 or an analogue thereof. In the present text, the parent peptide from which such a derivative is formally derived is in some places referred to as the “GLP-1 moiety” of the derivative.

In a preferred embodiment, the present invention relates to pharmaceutical composition according to claim 1, wherein the concentration of GLP-1 derivative is not less than 0.5 mg/ml, preferably not less than about 5 mg/ml, more preferred not less than about 10 mg/ml and, preferably, not more than about 100 mg/ml.

The pharmaceutical composition of the invention preferably comprises a GLP-1 derivative wherein at least one amino acid residue of the parent peptide has a lipophilic substituent attached. More preferred are compositions comprising a GLP-1 derivative having a lipophilic substituent which is attached to any one of the amino acid residues in position 18-38, preferably 26-34.

The pharmaceutical composition according to the invention, preferably further comprises one or more of the following substances:

- a pharmaceutically acceptable vehicle or carrier;
- an isotonic agent, preferably selected from the group consisting of sodium chloride, mannitol and glycerol;
- a preservative, preferably selected from the group consisting of phenol, m-cresol, methyl p-hydroxybenzoate, butyl p-hydroxybenzoate and benzyl alcohol;
- a buffer, preferably selected from the group consisting of sodium acetate, citrate, glycylglycine, histidine, 2-phenylethanol and sodium phosphate; and
- a surfactant capable of improving the solubility and/or the stability of the GLP-1 derivative, preferable selected from poloxymer 188, tween 20 and tween 80.

In a preferred embodiment, the pharmaceutical composition of the invention comprises a GLP-1 derivative wherein the lipophilic substituent comprises from 4 to 40 carbon atoms, preferably from 8 to 25 carbon atoms.

The lipophilic substituent is preferably attached to an amino acid residue in such a way that a carboxyl group of the lipophilic substituent forms an amide bond with an amino group of the amino acid residue, or, the lipophilic substituent is attached to an amino acid residue in such a way that an amino group of the lipophilic substituent forms an amide bond with a carboxyl group of the amino acid residue.

In a preferred embodiment the pharmaceutical composition according to the invention comprises a GLP-1 derivative wherein the lipophilic substituent is attached to the parent peptide by means of a spacer.

The spacer is preferably, in one embodiment, an unbranched alkane α,ω-dicarboxylic acid group having from 1 to 7 methylene groups, preferably two methylene groups, which form a bridge between an amino group of the parent peptide and an amino group of the lipophilic substituent.

The spacer is preferably, in another embodiment, an amino acid residue except Cys, or a dipeptide such as Gly-Lys or any unbranched alkane α,ω-aminoacid having from 1 to 7 methylene groups, preferably 2-4 methylene groups, which form a bridge between an amino group of the parent peptide and an amino group of the lipophilic substituent.

In a preferred embodiment, the lipophilic substituent comprises a partially or completely hydrogenated cyclopentanophenathrene skeleton.

In another preferred embodiment, the lipophilic substituent is a straight-chain or branched alkyl group.

The lipophilic substituent is preferably the acyl group of a straight-chain or branched fatty acid, the acyl group more preferably being:

- selected from the group comprising $CH_3(CH_2)_nCO$-, wherein n is 4 to 38, preferably $CH_3(CH_2)_6CO$-, $CH_3(CH_2)_8CO$-, $CH_3(CH_2)_{10}CO$-, $CH_3(CH_2)_{12}CO$-, $CH_3(CH_2)_{14}CO$-, $CH_3(CH_2)_{16}CO$-, $CH_3(CH_2)_{18}CO$-, $CH_3(CH_2)_{20}CO$- and $CH_3(CH_2)_{22}CO$-; or
- an acyl group of a straight-chain or branched alkane α,ω-dicarboxylic acid; or

- selected from the group comprising $HOOC(CH_2)_mCO$-, wherein m is from 4 to 38, preferably from 4 to 24, more preferred selected from the group comprising $HOOC(CH_2)_{14}CO$-, $HOOC(CH_2)_{16}CO$-, $HOOC(CH_2)_{18}CO$-, $HOOC(CH_2)_{20}CO$- and $HOOC(CH_2)_{22}CO$-.

In another preferred embodiment, the lipophilic substituent is a group of the formula $CH_3(CH_2)_p((CH_2)_qCOOH)CHNH\text{-}CO(CH_2)_2CO$-, wherein p and q are integers and p+q is an integer of from 8 to 33, preferably from 12 to 28.

In another preferred embodiment, the lipophilic substituent is a group of the formula $CH_3(CH_2)_rCO\text{-}NHCH(COOH)(CH_2)_2CO$-, wherein r is an integer of from 10 to 24.

In another preferred embodiment, the lipophilic substituent is a group of the formula $CH_3(CH_2)_sCO\text{-}NHCH((CH_2)_2COOH)CO$-, wherein s is an integer of from 8 to 24.

In another preferred embodiment, the lipophilic substituent is a group of the formula $\text{-}NHCH(COOH)(CH_2)_4NH\text{-}CO(CH_2)_uCH_3$, wherein u is an integer of from 8 to 18.

In another preferred embodiment, the lipophilic substituent is a group of the formula $\text{-}NHCH(COOH)(CH_2)_4NH\text{-}COCH((CH_2)_2COOH)NH\text{-}CO(CH_2)_wCH_3$, wherein w is an integer of from 10 to 16.

In another preferred embodiment, the lipophilic substituent is a group of the formula $\text{-}NHCH(COOH)(CH_2)_4NH\text{-}CO(CH_2)_2CH(COOH)NH\text{-}CO(CH_2)_xCH_3$, wherein x is an integer of from 10 to 16.

In another preferred embodiment, the lipophilic substituent is a group of the formula $\text{-}NHCH(COOH)(CH_2)_4NH\text{-}CO(CH_2)_2CH(COOH)NH\text{-}CO(CH_2)_yCH_3$, wherein y is zero or an integer of from 1 to 22.

In a preferred embodiment the pharmaceutical composition according to the invention, comprises a GLP-1 derivative wherein the parent peptide is GLP-1(A-B) wherein A is an integer from 1 to 7 and B is an integer from 38 to 45, or an analogue thereof.

The parent peptide is preferably, in one embodiment, selected from the group comprising GLP-1(7-35); GLP-1(7-36); GLP-1(7-36)amide; GLP-1(7-37); GLP-1(7-38); GLP-1(7-39); GLP-1(7-40) and GLP-1(7-41); and analogues thereof.

The parent peptide is preferably, in another embodiment, selected from the group comprising GLP-1(1-35); GLP-1(1-36); GLP-1(1-36)amide; GLP-1(1-37); GLP-1(1-38); GLP-1(1-39); GLP-1(1-40); GLP-1(1-41); and an analogues thereof.

In yet another embodiment, the parent peptide is a GLP-1 analogue of formula I:

```
 7   8   9   10  11  12  13  14  15  16  17
His-Xaa-Xaa-Gly-Xaa-Phe-Thr-Xaa-Asp-Xaa-Xaa-

18  19  20  21  22  23  24  25  26  27  28
Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Phe-

29  30  31  32  33  34  35  36  37  38
Ile-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa

39  40  41  42  43  44  45
Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa
                                  (I)
```

wherein
Xaa at position 8 is Ala, Gly, Ser, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 9 is Glu, Asp, or Lys,
Xaa at position 11 is Thr, Ala, Gly, Ser, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 14 is Ser, Ala, Gly, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 16 is Val, Ala, Gly, Ser, Thr, Leu, Ile, Tyr, Glu, Asp, or Lys,
Xaa at position 17 is Ser, Ala, Gly, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 18 is Ser, Ala, Gly, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 19 is Tyr, Phe, Trp, Glu, Asp, or Lys,
Xaa at position 20 is Leu, Ala, Gly, Ser, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 21 is Glu, Asp, or Lys,
Xaa at position 22 is Gly, Ala, Ser, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 23 is Gln, Asn, Arg, Glu, Asp, or Lys,
Xaa at position 24 is Ala, Gly, Ser, Thr, Leu, Ile, Val, Arg, Glu, Asp, or Lys,
Xaa at position 25 is Ala, Gly, Ser, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 26 is Lys, Arg, Gln, Glu, Asp, or His,

Xaa at position 27 is Glu, Asp, or Lys,
Xaa at position 30 is Ala, Gly, Ser, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 31 is Trp, Phe, Tyr, Glu, Asp, or Lys,
Xaa at position 32 is Leu, Gly, Ala, Ser, Thr, Ile, Val, Glu, Asp, or Lys,
Xaa at position 33 is Val, Gly, Ala, Ser, Thr, Met, Leu, Ile, Glu, Asp, or Lys,
Xaa at position 34 is Lys, Arg, Glu, Asp, or His,
Xaa at position 35 is Gly, Ala, Ser, Thr, Leu, Ile, Val, Glu, Asp, or Lys,
Xaa at position 36 is Arg, Lys, Glu, Asp, or His, or
Xaa at position 37 is Gly, Ala, Ser, Thr, Leu, Ile, Val, Glu, Asp, or Lys, or is deleted,
Xaa at position 38 is Arg, Lys, Glu, Asp, or His, or is deleted,
Xaa at position 39 is Arg, Lys, Glu, Asp, or His, or is deleted,
Xaa at position 40 is Asp, Glu, or Lys, or is deleted,
Xaa at position 41 is Phe, Trp, Tyr, Glu, Asp, or Lys, or is deleted,
Xaa at position 42 is Pro, Lys, Glu, or Asp, or is deleted,
Xaa at position 43 is Glu, Asp, or Lys, or is deleted,
Xaa at position 44 is Glu, Asp, or Lys, or is deleted, and
Xaa at position 45 is Val, Glu, Asp, or Lys, or is deleted, or

a C-1-6-ester, amide, C-1-6-alkylamide, C-1-6-dialkylamide and/or pharmaceutically acceptable salt thereof,
provided that

(i) when the amino acid at position 37, 38, 39, 40, 41, 42, 43 or 44 is deleted, then each amino acid downstream of the amino acid is also deleted,
(ii) the derivative of the GLP-1 analog contains only one Lys,
(iii) the ε-amino group of the Lys is substituted with a lipophilic substituent,
(iv) the total number of different amino acids between the derivative of the GLP-1 analog and the corresponding native form of GLP-1 does not exceed six.

The pharmaceutical composition according to the invention preferably comprises a GLP-1 derivative wherein a total of up to fifteen, preferably up to ten, more preferably up to six, amino acid residues have been exchanged with any α-amino acid residue which can be coded for by the genetic code.

The parent peptide is most preferably selected from one of the following the groups

- Arg^{26}-GLP-1(7-37); Arg^{34}-GLP-1(7-37); Lys^{36}-GLP-1(7-37); $Arg^{26,34}Lys^{36}$-GLP-1(7-37); $Arg^{26,34}Lys^{38}$GLP-1(7-38); $Arg^{26,34}Lys^{39}$-GLP-1(7-39); $Arg^{26,34}Lys^{40}$-GLP-1(7-40); $Arg^{26}Lys^{36}$-GLP-1(7-37); $Arg^{34}Lys^{36}$-GLP-1(7-37); $Arg^{26}Lys^{39}$-GLP-1(7-39); $Arg^{34}Lys^{40}$-GLP-1(7-40); $Arg^{26,34}Lys^{36,39}$-GLP-1(7-39); $Arg^{26,34}Lys^{36,40}$-GLP-1(7-40); Gly^{8}Arg26-GLP-1(7-37); $Gly^{8}Arg^{34}$-GLP-1(7-37); $Gly^{8}Lys^{36}$-GLP-1(7-37); $Gly^{8}Arg^{26,34}Lys^{36}$-GLP-1(7-37); $Gly^{8}Arg^{26,34}Lys^{39}$-GLP-1(7-39); $Gly^{8}Arg^{26,34}Lys^{40}$-GLP-1(7-40); $Gly^{8}Arg^{26}Lys^{36}$-GLP-1(7-37); $Gly^{8}Arg^{34}Lys^{36}$-GLP-1(7-37); $Gly^{8}Arg^{26}Lys^{39}$-GLP-1(7-39); $Gly^{8}Arg^{34}Lys^{40}$-GLP-1(7-40); $Gly^{8}Arg^{26,34}Lys^{36,39}$-GLP-1(7-39) and $Gly^{8}Arg^{26,34}Lys^{36,40}$-GLP-1(7-40); or

- $Arg^{26,34}Lys^{38}$GLP-1(7-38); $Arg^{26,34}Lys^{39}$GLP-1(7-39); $Arg^{26,34}Lys^{40}$GLP-1(7-40); $Arg^{26,34}Lys^{41}$GLP-1(7-41); $Arg^{26,34}Lys^{42}$GLP-1(7-42); $Arg^{26,34}Lys^{43}$GLP-1(7-43); $Arg^{26,34}Lys^{44}$GLP-1(7-44); $Arg^{26,34}Lys^{45}$GLP-1(7-45); $Arg^{26,34}Lys^{38}$GLP-1(1-38); $Arg^{26,34}Lys^{39}$GLP-1(1-39); $Arg^{26,34}Lys^{40}$GLP-1(1-40); $Arg^{26,34}Lys^{41}$GLP-1(1-41); $Arg^{26,34}Lys^{42}$GLP-1(1-42); $Arg^{26,34}Lys^{43}$GLP-1(1-43); $Arg^{26,34}Lys^{44}$GLP-1(1-44); $Arg^{26,34}Lys^{45}$GLP-1(1-45); $Arg^{26,34}Lys^{38}$GLP-1(2-38); $Arg^{26,34}Lys^{39}$GLP-1(2-39); $Arg^{26,34}Lys^{40}$GLP-1(2-40); $Arg^{26,34}Lys^{41}$GLP-1(2-41); $Arg^{26,34}Lys^{42}$GLP-1(2-42); $Arg^{26,34}Lys^{43}$GLP-1(2-43); $Arg^{26,34}Lys^{44}$GLP-1(2-44); $Arg^{26,34}Lys^{45}$GLP-1(2-45); $Arg^{26,34}Lys^{38}$GLP-1(3-38); $Arg^{26,34}Lys^{39}$GLP-1(3-39); $Arg^{26,34}Lys^{40}$GLP-1(3-40); $Arg^{26,34}Lys^{41}$GLP-1(3-41); $Arg^{26,34}Lys^{42}$GLP-1(3-42); $Arg^{26,34}Lys^{43}$GLP-1(3-43); $Arg^{26,34}Lys^{44}$GLP-1(3-44); $Arg^{26,34}Lys^{45}$GLP-1(3-45); $Arg^{26,34}Lys^{38}$GLP-1(4-38); $Arg^{26,34}Lys^{39}$GLP-1(4-39); $Arg^{26,34}Lys^{40}$GLP-1(4-40); $Arg^{26,34}Lys^{41}$GLP-1(4-41); $Arg^{26,34}Lys^{42}$GLP-1(4-42); $Arg^{26,34}Lys^{43}$GLP-1(4-43); $Arg^{26,34}Lys^{44}$GLP-1(4-44); $Arg^{26,34}Lys^{45}$GLP-1(4-45); $Arg^{26,34}Lys^{38}$GLP-1(5-38); $Arg^{26,34}Lys^{39}$GLP-1(5-39); $Arg^{26,34}Lys^{40}$GLP-1(5-40); $Arg^{26,34}Lys^{41}$GLP-1(5-41); $Arg^{26,34}Lys^{42}$GLP-1(5-42); $Arg^{26,34}Lys^{43}$GLP-1(5-43); $Arg^{26,34}Lys^{44}$GLP-1(5-44); $Arg^{26,34}Lys^{45}$GLP-1(5-45); $Arg^{26,34}Lys^{38}$GLP-1(6-38); $Arg^{26,34}Lys^{39}$GLP-1(6-39); $Arg^{26,34}Lys^{40}$GLP-1(6-40); $Arg^{26,34}Lys^{41}$GLP-1(6-41); $Arg^{26,34}Lys^{42}$GLP-1(6-42); $Arg^{26,34}Lys^{43}$GLP-1(6-43); $Arg^{26,34}Lys^{44}$GLP-1(6-44); $Arg^{26,34}Lys^{45}$GLP-1(6-45); $Arg^{26}Lys^{38}$GLP-1(1-38); $Arg^{34}Lys^{38}$GLP-1(1-38); $Arg^{26,34}Lys^{36,38}$GLP-1(1-38); $Arg^{26}Lys^{38}$GLP-1(7-38); $Arg^{34}Lys^{38}$GLP-1(7-38); $Arg^{26,34}Lys^{36,38}$GLP-1(7-38); $Arg^{26,34}Lys^{38}$GLP-1(7-38); $Arg^{26}Lys^{39}$GLP-1(1-39); $Arg^{34}Lys^{39}$GLP-1(1-39); $Arg^{26,34}Lys^{36,39}$GLP-1(1-39); $Arg^{26}Lys^{39}$GLP-1(7-39); $Arg^{34}Lys^{39}$GLP-1(7-39) and $Arg^{26,34}Lys^{36,39}$GLP-1(7-39).

The present invention furthermore relates to a method for improving the solubility and/or stability of GLP-1 or a fragment or an analogue thereof, characterised in that a lipophilic substituent is introduced on any one of the amino acid residues of the parent peptide.

By this method the lipophilic substituent is preferably introduced on any one of the amino acid residues in position 18-38, preferably 26-34.

The lipophilic substituent preferably comprises from 4 to 40 carbon atoms, more preferably from 8 to 25 carbon atoms.

In a preferred embodiment the lipophilic substituent is the acyl group of a straight-chain or branched fatty acid; preferably selected from the group comprising $CH_3(CH_2)_nCO$-, wherein n is 4 to 38, preferably $CH_3(CH_2)_6CO$-, $CH_3(CH_2)_8CO$-, $CH_3(CH_2)_{10}CO$-, $CH_3(CH_2)_{12}CO$-, $CH_3(CH_2)_{14}CO$-, $CH_3(CH_2)_{16}CO$-, $CH_3(CH_2)_{18}CO$-, $CH_3(CH_2)_{20}CO$- and $CH_3(CH_2)_{22}CO$-.

The GLP-1 parent peptide is preferably GLP-1(A-B) wherein A is an integer from 1 to 7 and B is an integer from 38 to 45, or an analogue thereof.

In one preferred embodiment, the GLP-1 is selected from the group comprising GLP-1(7-35); GLP-1(7-36); GLP-1(7-36)amide; GLP-1(7-37); GLP-1(7-38); GLP-1(7-39); GLP-1(7-40) and GLP-1(7-41); and analogues thereof.

In another preferred embodiment, the GLP-1 is selected from the group comprising GLP-1(1-35); GLP-1(1-36); GLP-1(1-36)amide; GLP-1(1-37); GLP-1(1-38); GLP-1(1-39); GLP-1(1-40); GLP-1(1-41); and an analogues thereof.

Brief description of the drawing

Figure 1: Circular Dichroism (CD) at 222 nm as a function of peptide concentration for acylated GLP-1 derivatives dissolved in 10 mM tris buffer, pH 8, and 23°C.

Detailed description of the invention

To obtain a satisfactory protracted profile of action of the GLP-1 derivative, the lipophilic substituent attached to the GLP-1 moiety preferably comprises 4-40 carbon atoms, in particular 8-25 carbon atoms. The lipophilic substituent may be attached to an amino group of the GLP-1

moiety by means of a carboxyl group of the lipophilic substituent which forms an amide bond with an amino group of the amino acid residue to which it is attached. Alternatively, the lipophilic substituent may be attached to said amino acid residue in such a way that an amino group of the lipophilic substituent forms an amide bond with a carboxyl group of the amino acid residue. As a further option, the lipophilic substituent may be linked to the GLP-1 moiety via an ester bond. Formally, the ester can be formed either by reaction between a carboxyl group of the GLP-1 moiety and a hydroxyl group of the substituent-to-be or by reaction between a hydroxyl group of the GLP-1 moiety and a carboxyl group of the substituent-to-be. As a further alternative, the lipophilic substituent can be an alkyl group which is introduced into a primary amino group of the GLP-1 moiety.

In one preferred embodiment of the invention, the lipophilic substituent is attached to the GLP-1 moiety by means of a spacer in such a way that a carboxyl group of the spacer forms an amide bond with an amino group of the GLP-1 moiety. Examples of suitable spacers are succinic acid, Lys, Glu or Asp, or a dipeptide such as Gly-Lys. When the spacer is succinic acid, one carboxyl group thereof may form an amide bond with an amino group of the amino acid residue, and the other carboxyl group thereof may form an amide bond with an amino group of the lipophilic substituent. When the spacer is Lys, Glu or Asp, the carboxyl group thereof may form an amide bond with an amino group of the amino acid residue, and the amino group thereof may form an amide bond with a carboxyl group of the lipophilic substituent. When Lys is used as the spacer, a further spacer may in some instances be inserted between the ε-amino group of Lys and the lipophilic substituent. In one preferred embodiment, such a further spacer is succinic acid which forms an amide bond with the ε-amino group of Lys and with an amino group present in the lipophilic substituent. In another preferred embodiment such a further spacer is Glu or Asp which forms an amide bond with the ε-amino group of Lys and another amide bond with a carboxyl group present in the lipophilic substituent, that is, the lipophilic substituent is a N^{ε}-acylated lysine residue.

In another preferred embodiment of the present invention, the lipophilic substituent has a group which can be negatively charged. One preferred group which can be negatively charged is a carboxylic acid group.

The parent peptide can be produced by a method which comprises culturing a host cell containing a DNA sequence encoding the polypeptide and capable of expressing the polypeptide in a

suitable nutrient medium under conditions permitting the expression of the peptide, after which the resulting peptide is recovered from the culture.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (*e.g.* in catalogues of the American Type Culture Collection). The peptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, *e.g.* ammonium sulphate, purification by a variety of chromatographic procedures, *e.g.* ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like, dependent on the type of peptide in question.

The DNA sequence encoding the parent peptide may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the peptide by hybridisation using synthetic oligonucleotide probes in accordance with standard techniques (see, for example, Sambrook, J, Fritsch, EF and Maniatis, T, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989). The DNA sequence encoding the peptide may also be prepared synthetically by established standard methods, *e.g.* the phosphoamidite method described by Beaucage and Caruthers, *Tetrahedron Letters* **22** (1981), 1859 - 1869, or the method described by Matthes *et al.*, *EMBO Journal* **3** (1984), 801 - 805. The DNA sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki *et al.*, *Science* **239** (1988), 487 - 491.

The DNA sequence may be inserted into any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, *i.e.* a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.* a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the peptide is operably linked to additional segments required for transcription of the DNA, such as a promoter. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the peptide of the invention in a variety of host cells are well known in the art, cf. for instance Sambrook *et al.*, *supra*.

The DNA sequence encoding the peptide may also, if necessary, be operably connected to a suitable terminator, polyadenylation signals, transcriptional enhancer sequences, and translational enhancer sequences. The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, *e.g.* a gene the product of which complements a defect in the host cell or one which confers resistance to a drug, *e.g.* ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate.

To direct a parent peptide of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the peptide in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that normally associated with the peptide or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the present peptide, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook *et al..*, *supra*).

The host cell into which the DNA sequence or the recombinant vector is introduced may be any cell which is capable of producing the present peptide and includes bacteria, yeast, fungi and higher eukaryotic cells. Examples of suitable host cells well known and used in the art are, without limitation, *E. coli*, *Saccharomyces cerevisiae*, or mammalian BHK or CHO cell lines.

Examples of compounds which can be useful as GLP-1 moieties according to the present invention are described in International Patent Application No. WO 87/06941 (The General Hospital Corporation) which relates to a peptide fragment which comprises GLP-1(7-37) and functional derivatives thereof and to its use as an insulinotropic agent.

Further GLP-1 analogues are described in International Patent Application No. 90/11296 (The General Hospital Corporation) which relates to peptide fragments which comprise GLP-1(7-36) and functional derivatives thereof and have an insulinotropic activity which exceeds the insulinotropic activity of GLP-1(1-36) or GLP-1(1-37) and to their use as insulinotropic agents.

International Patent Application No. 91/11457 (Buckley *et al.*.) discloses analogues of the active GLP-1 peptides 7-34, 7-35, 7-36, and 7-37 which can also be useful as GLP-1 moieties according to the present invention.

Preparation and administration of the compositions

Pharmaceutical compositions containing a GLP-1 derivative according to the present invention may be administered parenterally or orally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the GLP-1 derivative in the form of a nasal or pulmonal spray. As a still further option, the pharmaceutical compositions containing a the GLP-1 derivatives of the invention can also be adapted to transdermal administration, *e.g.* from a patch, optionally a iontophoretic patch, or transmucosal, *e.g.* bucal, administration.

Pharmaceutical compositions containing a GLP-1 derivative of the present invention may be prepared by conventional techniques, *e.g.* as described in Remington's *Pharmaceutical Sciences*, 1985 or in Remington: *The Science and Practice of Pharmacy*, 19^{th} edition, 1995.

Thus, the injectable compositions of the GLP-1 derivative of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

According to one procedure, the GLP-1 derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, *e.g.* hydrochloric acid, or a base, *e.g.* aqueous sodium hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

A composition for nasal administration of certain peptides may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S) or in WO 93/18785.

According to one preferred embodiment of the present invention, the pharmaceutical compositions containing a GLP-1 derivative is provided in the form of a composition suitable for administration by injection. Such a composition can either be an injectable solution ready for use or it can be an amount of a solid composition, *e.g.* a lyophilised product, which has to be dissolved in a solvent before it can be injected. The injectable solution preferably contains not less than about 0.5 mg/ml, preferably not less than about 5 mg/ml, more preferred not less than about 10 mg/ml of the GLP-1 derivative and, preferably, not more than about 100 mg/ml of the GLP-1 derivative.

The pharmaceutical compositions containing a GLP-1 derivative of this invention can be used in the treatment of various diseases. The particular GLP-1 derivative to be used and the optimal dose level for any patient will depend on the disease to be treated and on a variety of factors including the efficacy of the specific peptide derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case. It is recommended that the dosage of the GLP-1 derivative of this invention be determined for each individual patient by those skilled in the art.

In particular, it is envisaged that the pharmaceutical compositions containing a GLP-1 derivative will be useful for the treatment of non-insulin dependent diabetes mellitus and/or for the treatment of obesity.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection.

Examples

Circular Dichroism (CD) at 222 nm as a function of peptide concentration for peptides dissolved in 10 mM tris buffer, pH 8, and 23°C was measured for GLP-1(7-37) and the following eight GLP-1 derivatives suitable for pharmaceutical formulations according to the present invention:

Derivative	*Position of lipophilic substituent*
(a)	26
(b)	26
(c)	23
(d)	34
(e)	38
(f)	26
(g)	26
(h)	18

(a) is $Arg^{34}Lys^{26}(N^{\varepsilon}$-($\gamma$-glutamyl($N^{\alpha}$-hexadecanoyl)))-GLP-1(7-37)-OH;

(b) is Arg^{34},Lys^{26} (N^{ε}-(γ-glutamyl(N^{α}-lithochoyl))) GLP-1(7-37)-OH;

(c) is $Arg^{26,34}$,Lys^{23} (N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37)-OH;

(d) is Arg^{26},Lys^{34} (N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1(7-37)-OH;

(e) is Gly^{8},Glu^{37},$Arg^{26,34}$,Lys^{38} (N^{ε}-(γ-glutamyl(N^{α}-octadecanoyl))) GLP-1(7-38)-OH;

(f) is Arg^{34},Lys^{26} (N^{ε}-(hexadecanoyl)) GLP-1 (7-37)-OH;

(g) is Arg^{34},Lys^{26} (N^{ε}-(γ-aminobutyroyl(N^{γ}-hexadecanoyl))) GLP-1 (7-37)-OH;

(h) is $Arg^{26,34}$,Lys^{18} (N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37)-OH.

The results are presented in Figure 1. Note that the CD signal is proportional to the average content of α-helix in the peptides, i.e., a CD value of -1 corresponds to 10% α-helix content under these conditions. The figure shows that, as the concentration of unmodified GLP-1(7-37)

is raised between 25 and 1000 μM, the content of α-helix increases form about 15% to about 30-35% in parallel with the formation of higher oligomers. In contrast with this concentration dependent behaviour, the figure shows that the helix content remains high and essentially independent of the concentration in the 1-200 μM range for a series of acylated glp-1 derivatives forming partially structured micelle-like aggregates under the same conditions.

The following acronyms for commercially available chemicals are used:

DMF	:	N,N-Dimethylformamide.
DCC	:	N,N-Dicyclohexylcarbodiimide
NMP	:	N-Methyl-2-pyrrolidone.
EDPA	:	N-Ethyl-N,N-diisopropylamine.
EGTA	:	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
GTP		Guanosine 5'-triphosphate.
TFA	:	Trifluoroacetic acid.
THF	:	Tetrahydrofuran
H-Glu(OH)-OBu^t:		L-Glutamic acid α-tert-butyl ester
Cap-ONSu:		Octanoic acid 2,5-dioxopyrrolidin-1-yl ester
Lau-ONSu:		Dodecanoic acid 2,5-dioxopyrrolidin-1-yl ester
Myr-ONSu:		Tetradecanoic acid 2,5-dioxopyrrolidin-1-yl ester.
Pal-ONSu:		Hexadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.
Ste-ONSu		Octadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

Abbreviations:

PDMS: Plasma Desorption Mass Spectrometry

MALDI-MS: Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry

HPLC: High Performance Liquid Chromatography

amu: atomic mass units

Lit-Glu(ONSu)-OBu^t: N$^\alpha$-Lithochoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Cap-Glu(ONSu)-OBu^t: N$^\alpha$-Octanoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Cac-Glu(ONSu)-OBu^t: N$^\alpha$-Decanoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Lau-Glu(ONSu)-OBu^t: N$^\alpha$-Dodecanoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Myr-Glu(ONSu)-OBu^t: N$^\alpha$-Tetradecanoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Pal-Glu(ONSu)-OBu^t : N$^\alpha$-Hexadecanoyl-(L)-glutamic acid α-t-butyl-γ-2,5-dioxopyrrolidin-1-yl diester.

Ste-Glu(ONSu)-OBu^t : N$^\alpha$-Octadecanoyl-(L)-glutamic acid α-t-butyl-γ-2,5-dioxopyrrolidin-1-yl diester

Lau-β-Ala-ONSu: N$^\beta$-Dodecanoyl-β-alanine 2,5-dioxopyrrolidin-1-yl ester

Pal-β-Ala-ONSu: N$^\beta$-Hexadecanoyl-β-alanine 2,5-dioxopyrrolidin-1-yl ester

Lau-GABA-ONSu: N$^\gamma$-Dodecanoyl-γ-aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Myr-GABA-ONSu: N$^\gamma$-Tetradecanoyl-γ-aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Pal-GABA-ONSu: N$^\gamma$-Hexadecanoyl-γ-aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Ste-GABA-ONSu: N$^\gamma$-Octadecanoyl-γ-aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Pal-Isonip-ONSu: N-Hexadecanoyl-piperidine-4-carboxylic acid 2,5-dioxopyrrolidin-1-yl ester

Pal-Glu(OBu^t)-ONSu: N$^\alpha$-Hexadecanoyl-L-glutamic acid α-2,5-dioxopyrrolidin-1-yl ester γ-t-butyl ester

HOOC-$(CH_2)_6$-COONSu: ω–Carboxyheptanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{10}$-COONSu: ω–Carboxyundecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{12}$-COONSu: ω–Carboxytridecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{14}$-COONSu: ω–Carboxypentadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{16}$-COONSu: ω–Carboxyheptadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{18}$-COONSu: ω–Carboxynonadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

Analytical

Plasma Desorption Mass Spectrometry

Sample preparation:

The sample is dissolved in 0.1 % TFA/EtOH (1:1) at a concentration of 1 µg/µl. The sample solution (5-10 µl) is placed on a nitrocellulose target (Bio-ion AB, Uppsala, Sweden) and allowed to adsorb to the target surface for 2 minutes. The target is subsequently rinsed with 2x25 µl 0.1 % TFA and spin-dried. Finally, the nitrocellulose target is placed in a target carrousel and introduced into the mass spectrometer.

MS analysis:

PDMS analysis was carried out using a Bio-ion 20 time-of flight instrument (Bio-ion Nordic AB, Uppsala, Sweden). An acceleration voltage of 15 kV was applied and molecular ions formed by bombardment of the nitrocellulose surface with 252-Cf fission fragments were accelerated towards a stop detector. The resulting time-of-flight spectrum was calibrated into a true mass spectrum using the H^+ and NO^+ ions at m/z 1 and 30, respectively. Mass spectra were generally accumulated for 1.0×10^6 fission events corresponding to 15-20 minutes. Resulting assigned masses all correspond to isotopically averaged molecular masses. The accuracy of mass assignment is generally better than 0.1 %.

MALDI-MS

MALDI-TOF MS analysis was carried out using a Voyager RP instrument (PerSeptive Biosystems Inc., Framingham, MA) equipped with delayed extraction and operated in linear mode. Alpha-cyano-4-hydroxy-cinnamic acid was used as matrix, and mass assignments were based on external calibration.

Further examples of compounds which can be useful as GLP-1 derivatives in the pharmaceutical composition according to the present invention are

Example 1

Synthesis of $Arg^{26,34}$, Lys^{36} (N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-36)-OH.

To a mixture of $Arg^{26,34}$, Lys^{36} GLP-1 (7-36)-OH (12.2 mg, 3.67 μmol), EDPA (13.3 mg, 103 μmol), NMP (1.71 ml) and water (855 μl) was added a solution of Pal-Glu(ONSu)-OBu^t (5.94 mg, 11 μmol), prepared as described in PCT application no. PCT/DK97/00340, in NMP (148 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (6 mg, 81 μmol) in water (0.6 ml). A 0.5 % aqueous solution of ammonium-acetate (38 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (20 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The <u>title compound</u> (3.1 mg, 23 %) was isolated, and the product was analysed by PDMS. The m/z

value for the protonated molecular ion was found to be 3695 +- 3. The resulting molecular weight is thus 3694 +- 3 amu (theoretical value 3694 amu).

Example 2

Synthesis of $Arg^{26,34}$,Lys^{36} (N^{ε}-(γ-glutamyl(N^{α}-octadecanoyl))) GLP-1 (7-36)-OH.

To a mixture of $Arg^{26,34}$,Lys^{36} GLP-1 (7-36)-OH (12.2 mg, 3.7 µmol), EDPA (13.3 mg, 103 µmol), NMP (1.71 ml) and water (855 µl) was added a solution of Ste-Glu(ONSu)-OBu^t (6.25 mg, 11 µmol), prepared as described in PCT application no. PCT/DK97/00340, in NMP (1 ml). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (6 mg, 81 µmol) in water (0.6 ml). A 0.5 % aqueous solution of ammonium acetate (54 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (20 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (3.7 mg, 27 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3723 +- 3. The resulting molecular weight is thus 3722 +- 3 amu (theoretical value 3722 amu).

Example 3

Synthesis of lithocholic acid 2,5-dioxopyrrolidin-1-yl ester.

To a solution of lithocholic acid (5.44 g, 14.3 mmol) in a mixture of anhydrous THF (120 ml) and anhydrous acetonitril (30 ml) was added N-hydroxysuccinimide (1.78 g, 15 mmol). The mixture was cooled to 10°C, a solution of DCC (3.44 g, 16.7 mmol) in anhydrous THF (30 ml) was added drop wise, and the resulting reaction mixture stirred for 16 h at room temperature. The reaction mixture was filtered and partitioned between dichloromethane (450 ml) and 10% aqueous Na_2CO_3 (150 ml). The phases were separated, and the organic phase washed with 10% aqueous Na_2CO_3 (150 ml), water (2x150 ml), and dried ($MgSO_4$). The solvent was concentrated *in vacuo*. The residue was crystallised from a mixture of dichloromethane (30 ml) and n-heptane (30 ml). The precipitate was dried in a vacuum drying oven for 36 h to give the title compound (3.46 g, 51 %).

Example 4

Synthesis of Lit-Glu(ONSu)-OBu^t.

A suspension of H-Glu(OH)-OBu^t (1.28 g, 6.33 mmol), DMF (88 ml) and EDPA (0.82 g, 6.33 mmol) and lithocholic acid 2,5-dioxopyrrolidin-1-yl ester, prepared as described in example 3, was stirred for 16 h at room temperature. The reaction mixture was concentrated *in vacuo* and the residue dissolved in ethyl acetate (40 ml). The resulting solution was washed with 5% aqueous citric acid (2x25 ml), brine (10 ml), and filtered). The solvent was concentrated *in vacuo* and the residue dissolved in DMF (12 ml). The resulting solution was added drop wise to a 10% aqueous solution of citric acid whereby the product precipitates. The precipitate was collected and washed with iced water, and dried *in vacuo*. The crude product was recrystallised from a mixture of n-heptane (40 ml) and 2-propanol (17 ml). The precipitate was dried in a vacuum drying oven for 4 h to give the free acid intermediate. To a solution of the free acid intermediate in DMF (18 ml) was added hydroxysuccinimide (0.45 g, 3.91 mmol), followed by a solution of DCC (0.73 g, 3.56 mmol) in dichloromethane (18 ml). The resulting mixture was stirred at ambient temperature for 18 h, and then filtered. The filtrate was concentrated *in vacuo* to a solid, and the residue was dissolved in dichloromethane (25 ml), and the filtration repeated, the solvent removed *in vacuo* to give a foam. The residue was dissolved in refluxing n-heptane (35 ml), and the product crystallised b addition of 2-propanol. The precipitate was collected, washed with cold n-heptane, dried at 35°C *in vacuo* to give the <u>title compound</u> (1.34 g, 57%).

Example 5

Synthesis of Arg^{34},Lys^{26} (N$^\varepsilon$-(γ-glutamyl(N$^\alpha$-lithochoyl))) GLP-1 (7-37)-OH.

To a mixture of Arg^{34},Lys^{26} GLP-1 (7-37)-OH (41.1 mg, 12.2 µmol), EDPA (44 mg, 340 µmol), NMP (5.76 ml) and water (2.88 ml) was added a solution of Lit-Glu(ONSu)-OBu^t (24 mg, 37 µmol), prepared as described in example 4, in NMP (600 µl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 75 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20 mg, 268 µmol) in water (2 ml). A 0.5 % aqueous solution of ammonium acetate (128 ml) was added, and the resulting mixture divided into two equal portions, and each portion eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (2x25 ml), and finally liberated from the cartridge by elution with TFA (2x25 ml). The combined eluates were concentrated *in vacuo*, and the residue purified by column chromato-graphy using a cyanopropyl column (Zorbax 300SB-CN) and a stan-

dard acetonitril/TFA system. The column was heated to 65^{O}C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (5 mg, 11 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3872 +- 3. The resulting molecular weight is thus 3871 +- 3 amu (theoretical value 3871 amu).

Example 6

Synthesis of Arg^{26},Lys^{34} (N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37)-OH

To a mixture of Arg^{26},Lys^{34} GLP-1 (7-37)-OH (18 mg, 5.3 μmol), EDPA (19.3 mg, 149 μmol), NMP (2.52 ml) and water (1.26 ml) was added a solution of Pal-Glu(ONSu)-OBu^{t} (8.6 mg, 16 μmol) in NMP (215 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (8.8 mg, 117 μmol) in water (0.88 ml). A 0.5 % aqueous solution of ammonium acetate (50 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65^{O}C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (6 mg, 30 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3752 +- 3. The resulting molecular weight is thus 3751 +- 3 amu (theoretical value 3751 amu).

Example 7

Synthesis of desamino-His^{7},Arg^{26},Lys^{34} (N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37)-OH.

To a mixture of desamino-His^{7},Arg^{26},Lys^{34} GLP-1 (7-37)-OH (14.3 mg, 4.2 μmol), EDPA (15.3 mg, 119 μmol), NMP (2 ml) and water (1 ml) was added a solution of Pal-Glu(ONSu)-OBu^{t} (6.84 mg, 12.7 μmol) in NMP (171 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 50 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (7 mg, 99 μmol) in water (700 μl). A 0.5 % aqueous solution of ammonium acetate (42 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue

purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65^{o}C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (5.6 mg, 35 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3738 +- 3. The resulting molecular weight is thus 3737 +- 3 amu (theoretical value 3737 amu).

Example 8

Synthesis of Gly^{8},Arg^{26,34},Lys^{38} (N$^{\varepsilon}$-(γ-glutamyl(N$^{\alpha}$-hexadecanoyl))) GLP-1 (7-38)-OH.

To a mixture of Gly^{8},Arg^{26,34},Lys^{38} GLP-1 (7-38)-OH (11.8 mg, 3.4 µmol), EDPA (12.1 mg, 94 µmol), NMP (1.65 ml) and water (0.83 ml) was added a solution of Pal-Glu(ONSu)-OBu^{t} (5.4 mg, 10 µmol) in NMP (135 µl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 75 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.5 mg, 73.7 µmol) in water (553 µl). A 0.5 % aqueous solution of ammonium acetate (36 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65^{o}C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (5 mg, 38 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3895 +- 3. The resulting molecular weight is thus 3894 +- 3 amu (theoretical value 3894 amu).

Example 9

Synthesis of Gly^{8},Glu^{37},Arg^{26,34},Lys^{38} (N$^{\varepsilon}$-(γ-glutamyl(N$^{\alpha}$-hexadecanoyl))) GLP-1 (7-38)-OH.

To a mixture of Gly^{8},Glu^{37},Arg^{26,34},Lys^{38} GLP-1 (7-38)-OH (9 mg, 2.48 µmol), EDPA (9 mg, 69.4 µmol), NMP (1.25 ml) and water (0.63 ml) was added a solution of Pal-Glu(ONSu)-OBu^{t} (4 mg, 7.4 µmol) in NMP (100 µl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 105 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (4.1 mg, 54.6 µmol) in water (410 µl). A 0.5 % aqueous solution of ammonium acetate (27 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobi-

lised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by elution with TFA (15 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (2.9 mg, 29 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3967 +- 3. The resulting molecular weight is thus 3966 +- 3 amu (theoretical value 3967 amu).

Example 10

Synthesis of Gly^{8},Glu^{37},$Arg^{26,34}$,Lys^{38} (N^{ε}-(γ-glutamyl(N^{α}-octadecanoyl))) GLP-1 (7-38)-OH.

To a mixture of Gly^{8},Glu^{37},$Arg^{26,34}$,Lys^{38} GLP-1 (7-38)-OH (9 mg, 2.5 μmol), EDPA (9 mg, 69.4 μmol), NMP (1.25 ml) and water (0.63 ml) was added a solution of Ste-Glu(ONSu)-OBu^{t} (4.2 mg, 7.4 μmol in NMP (105 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 105 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (4.1 mg, 54.6 μmol) in water (409 μl). A 0.5 % aqueous solution of ammonium acetate (27 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by elution with TFA (15 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (3.2 mg, 32 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3995 +- 3. The resulting molecular weight is thus 3994 +- 3 amu (theoretical value 3995 amu).

Example 11

Synthesis of Cap-Glu(ONSu)-OBu^{t}.

To a solution of octanoic acid (5 g, 34.7 mmol) and N-hydroxysuccinimide (4 g, 34.7 mmol) in anhydrous acetonitril (10 ml) was added a solution of DCC (7.15 g, 34.7 mmol) in anhydrous dichloromethane (15 ml), and the resulting reaction mixture stirred for 16 h at room temperature. The precipitated solid was filtered off and recrystallised from a mixture of n-heptane (40 ml) and 2-propanol (2 ml). The precipitate was dried in a vacuum drying oven for 16 h to give the intermediate Cap-ONSu. A suspension of the crude ester intermediate

(3.9 g, 16.2 mmol), (L)-H-Glu(OH)-OBu^t (3.28 g, 16.2 mmol), DMF (268 ml) and EDPA (2.1 g, 16.2 mmol) was stirred for 64 h at room temperature. The reaction mixture was concentrated *in vacuo* and the residue dissolved in ethyl acetate (50 ml). The resulting solution was washed with 5% aqueous citric acid (2x25 ml). The solvent was concentrated *in vacuo* and the residue dissolved in DMF (36 ml). The resulting solution was added drop wise to a 10% aqueous solution of citric acid (357 ml) and extracted with ethyl acetate (200 ml), and dried ($MgSO_4$). The solvent was concentrated *in vacuo* to give the crude glutamic acid intermediate. To a mixture of the crude glutamic acid intermediate, N-hydroxysuccinimide (1.85 g, 16.1 mmol) and DMF (25 ml) was added a solution of DCC (3.32 g, 16.1 mmol) in dichloromethane (15 ml). The resulting mixture was stirred at ambient temperature for 20 h. The reaction mixture was filtered and the solvent concentrated *in vacuo*. The residue was purified on a silica gel column (40- 63μ), eluted with a mixture of dichloromethane and acetonitril (1:1) to give the title compound (0.63 g, 6% over all).

Example 12

Synthesis of desamino-His^7,Arg^{26},Lys^{34} (N$^\varepsilon$-(γ-glutamyl(N$^\alpha$-octanoyl))) GLP-1 (7-37)-OH.

To a mixture of desamino-His^7,Arg^{26},Lys^{34} GLP-1 (7-37)-OH (14.3 mg, 4.2 μmol), EDPA (15.3 mg, 119 μmol), NMP (2 ml) and water (1 ml) was added a solution of Cap-Glu(ONSu)-OBu^t. (6.8 mg, 12.7 μmol), prepared as described in example 11, in NMP (135 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (7 mg, 93 μmol) in water (698 μl). A 0.5 % aqueous solution of ammonium acetate (42 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (4.1 mg, 27 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3626 +- 3. The resulting molecular weight is thus 3625 +- 3 amu (theoretical value 3625 amu).

Example 13

Synthesis of Glu^{37},Arg^{26,34},Lys^{38} (N$^\varepsilon$-(γ-glutamyl(N$^\alpha$-hexadecanoyl))) GLP-1 (7-38)-OH.

To a mixture of Glu^{37},$Arg^{26,34}$,Lys^{38} GLP-1 (7-38)-OH (17.6 mg, 4.9 μmol), EDPA (17.6 mg, 136 μmol), NMP (1.23 ml) and water (2.46 ml) was added a solution of Pal-Glu(ONSu)-OBu^t (7.9 mg, 14.6 μmol) in NMP (197 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (8 mg, 107 μmol) in water (804 μl). A 0.5 % aqueous solution of ammonium acetate (49 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (5.1 mg, 26 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3981 +- 3. The resulting molecular weight is thus 3980 +- 3 amu (theoretical value 3981 amu).

Example 14

Synthesis of Arg^{34},Lys^{26} (N^{ε}-(γ-glutamyl(N^{α}-octadecanoyl))) GLP-1 (7-37)-OH.

To a mixture of Arg^{34} GLP-1 (7-37)-OH (41.1 mg, 12.2 μmol), EDPA (44 mg, 341 μmol), NMP (5.76 ml) and water (2.88 ml) was added a solution of Ste-Glu(ONSu)-OBu^t (20.7 mg, 36.5 μmol in NMP (517 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 2 h at room temperature. The reaction was quenched by the addition of a solution of glycine (20.1 mg, 268 μmol) in water (2.01 ml). A 0.5 % aqueous solution of ammonium-acetate (120 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (15.4 mg, 34 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3781 +- 3. The resulting molecular weight is thus 3780 +- 3 amu (theoretical value 3779 amu).

Example 15

Synthesis of Arg^{34},Lys^{26}(N$^{\varepsilon}$-decanoyl) GLP-1 (7-37).

To a mixture of Arg^{34}-GLP-1 (7-37)-OH (20 mg, 5.9 µmol), EDPA (21.4 mg, 165 µmol), NMP (2.8 ml) and water (1.4 ml) was added a solution of Cac-ONSu (4.8 mg, 17.7 µmol) in NMP (119 µl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 2h at room temperature. The reaction was quenched by the addition of a solution of glycine (9.8 mg, 130 µmol) in water (98 µl). The resulting mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (7.4 mg, 35%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3539.6 ± 3. The resulting molecular weight is thus 3538.6 ± 3 amu (theoretical value 3538 amu).

Example 16

Synthesis of Arg^{34},Lys^{26} (N$^{\varepsilon}$-(hexadecanoyl)) GLP-1 (7-37)-OH.

To a mixture of Arg^{34} GLP-1 (7-37)-OH (41.1 mg, 12.2 µmol), EDPA (44 mg, 340 µmol), NMP (2.88 ml) and water (2.88 ml) was added a solution of Pal-ONSu (12.9 mg, 36.5 µmol) in NMP (3.3 ml). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 110 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20.1 mg, 268 µmol) in water (201 µl). The solvent was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (15 mg, 34 %) was isolated, and the product was analysed by PDMS.

Example 17

Synthesis of Arg^{26,34},Lys^{27} (N$^{\varepsilon}$-(γ-glutamyl(N$^{\alpha}$-hexadecanoyl))) GLP-1 (7-37)-OH

To a mixture of Arg^{26,34}, Lys^{27} GLP-1 (7-37)-OH (11.6 mg, 3.4 µmol), EDPA (12.3 mg, 94.9 µmol), NMP (1.6 ml) and water (0.8 ml) was added a solution of Pal-Glu(ONSu)-OBu^{t} (5.5 mg, 10.2 µmol) in NMP (137 µl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.6 mg, 74.6 µmol) in water (560 µl). A 0.5 % aqueous solution of ammonium acetate (34 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by eluti-

on with TFA (25 ml). The solvent was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (2.1 mg, 16 %) was isolated, and the product was analysed by PDMS.

Example 18

Synthesis of $Arg^{26,34}$,Lys^{23} (N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37)-OH.

To a mixture of $Arg^{26,34}$, Lys^{23} GLP-1 (7-37)-OH (11.6 mg, 3.4 μmol), EDPA (12.3 mg, 94.9 μmol), NMP (1.6 ml) and water (0.8 ml) was added a solution of Pal-Glu(ONSu)-OBu^t (5.5 mg, 10.2 μmol) in NMP (137 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.6 mg, 74.6 μmol) in water (560 μl). A 0.5 % aqueous solution of ammonium acetate (34 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (15 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The solvent was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (3.1 mg, 24 %) was isolated, and the product was analysed by PDMS.

Example 19

Synthesis of $Arg^{26,34}$,Lys^{18} (N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37)-OH

To a mixture of $Arg^{26,34}$, Lys^{18} GLP-1 (7-37)-OH (11.7 mg, 3.4 μmol), EDPA (12.2 mg, 94.6 μmol), NMP (1.6 ml) and water (0.8 ml) was added a solution of Pal-Glu(ONSu)-OBu^t (5.5 mg, 10.2 μmol) in NMP (137 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (5.6 mg, 74.6 μmol) in water (560 μl). A 0.5 % aqueous solution of ammonium acetate (34 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The solvent was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard

acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (1.9 mg, 15 %) was isolated, and the product was analysed by PDMS.

Example 20

Synthesis of Arg^{34},Lys^{26} (N^{ε}-(octanoyl)) GLP-1 (7-37)-OH.

To a mixture of Arg^{34} GLP-1 (7-37)-OH (41.1 mg, 12.2 μmol), EDPA (44 mg, 341 μmol), NMP (5.76 ml) and water (2.88 ml) was added a solution of Cap-ONSu (8.8 mg, 36.5 μmol, prepared as described in example 11, in NMP (106 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 115 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20 mg, 268 μmol) in water (200 μl). The solvent was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (18.8 mg, 44 %) was isolated, and the product was analysed by PDMS.

Example 21

Synthesis of Arg^{34},Lys^{26} (N^{ε}-(dodecanoyl)) GLP-1 (7-37)-OH.

To a mixture of Arg^{34} GLP-1 (7-37)-OH (41.1 mg, 12.2 μmol), EDPA (44 mg, 341 μmol), NMP (5.76 ml) and water (2.88 ml) was added a solution of Lau-ONSu (8.8 mg, 36.5 μmol, prepared in a similar manner as described for Cap-ONSu in example 11), in NMP (271 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 100 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20.1 mg, 268 μmol) in water (200 μl). The solvent was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (18 mg, 42 %) was isolated, and the product was analysed by PDMS.

Example 22

Synthesis of Pal-GABA-ONSu.

A mixture of Pal-ONSu (3 g, 8.48 mmol), γ-aminobutyric acid (0.87 g, 8.48 mmol) in DMF (200 ml) was stirred at room temperature for 60 h. The reaction mixture was filtered

and the filtrate was added drop wise to 10% aqueous citric acid (500 ml). The precipitated N-acylated intermediate was collected and dried *in vacuo*. To a suspension of the dried intermediate in DMF (35 ml) was added a solution of DCC (1.45 g, 7.0 mmol) in dichloromethane (20 ml). The resulting mixture was stirred at room temperature for 20 h, and then filtered. The solvent was removed *in vacuo* to give a solid residue. The residue was recrystallised from a mixture of n-heptane (50 ml) and 2-propanol (2.5 ml) to give the title compound (2.5 g, 75 %).

Example 23

Synthesis of Arg^{34},Lys^{26} (N^{ε}-(γ-aminobutyroyl(N^{γ}-hexadecanoyl))) GLP-1 (7-37)-OH.

To a mixture of Arg^{34}, Lys^{26} GLP-1 (7-37)-OH (41.1 mg, 12.2 μmol), EDPA (44 mg, 341 μmol), NMP (5.76 ml) and water (2.88 ml) was added a solution of Pal-GABA-ONSu (16 mg, 36.5 μmol, prepared as described in example 22) in NMP (400 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 100 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20 mg, 268 μmol) in water (200 μl). The solvent was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (15.8 mg, 35 %) was isolated, and the product was analysed by PDMS.

Example 24

Synthesis of N^{α}-hexadecanoyl-D-glutamic acid α-t-butyl ester-γ-2,5-dioxopyrrolidin-1-yl ester.

A mixture of Pal-ONSu (6.64 g, 18.8 mmol), D-glutamic acid α-tert-butyl ester (4.5 g, 18.8 mmol) and EDPA (4.85 g, 37.5 mmol) in DMF (538 ml) was stirred at room temperature for 60 h. The solvent was removed and the residue dissolved in ethyl acetate (175 ml). The resulting solution was extracted with 10% aqueous citric acid (2x125 ml), and the organic phase concentrated *in vacuo*. The residue was dissolved in DMF (60 ml), and the resulting mixture slowly added to 10% aqueous citric acid (500 ml). The precipitated compound was collected and dried *in vacuo*, to give the crude N-acylated glutamic acid intermediate. The crude intermediate was dissolved in DMF (35 ml), and a solution of DCC (3.5 g, 17 mmol) in dichloromethane (70 ml) was added. The resulting mixture was stirred at room temperature for 20 h, and then filtered. The filtrate was concentrated *in vacuo*, and the solid

residue recrystallised from a mixture of n-heptane (75 ml) and 2-propanol (5 ml), to give the title compound (5.2 g, 50 %)

Example 25

Synthesis of Arg^{34},Lys^{26} (N^{ε}-(γ-D-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37)-OH.

To a mixture of Arg^{34}, Lys^{26} GLP-1 (7-37)-OH (41.1 mg, 12.2 μmol), EDPA (44 mg, 341 μmol), NMP (5.76 ml) and water (2.88 ml) was added a solution of N^{α}-hexadecanoyl-D-glutamic acid α-t-butyl ester-γ-2,5-dioxopyrrolidin-1-yl ester (19.7 mg, 36.5 μmol) in NMP (491 μl). The reaction mixture was gently shaken for 5 min. at room temperature, and then allowed to stand for an additional 95 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (20 mg, 268 μmol) in water (2 ml). A 0.5 % aqueous solution of ammonium acetate (120 ml) was added, and the resulting mixture divided into to equal portions, and each portion eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The combined eluates were concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (10.5 mg, 23 %) was isolated, and the product was analysed by PDMS.

Example 26

Synthesis of Lys^{34} (N^{ε}-(γ-glutamyl(N^{α}-tetradecanoyl))) GLP-1 (7-37).

To a mixture of GLP-1 (7-37)-OH (33.6 mg, 8.9 μmol), EDPA (32.4 mg, 250 μmol), NMP (2.1 ml) and water (2.1 ml) was added a solution of Myr-Glu(ONSu)-OBu^t (9.1 mg, 17.9 μmol), prepared as described in PCT application no. PCT/DK97/00340, in NMP (228 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 80 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (14.8 mg, 197 μmol) in water (1.47 ml). A 0.5% aqueous solution of ammonium acetate (100 ml) was added, and the resulting mixture divided into two equal portions, and each portion eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (2x25 ml), and finally liberated from the cartridge by elution with TFA (2x25 ml). The combined eluates were concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-

100% in 60 minutes. The title compound (0.19 mg, 0.6%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3693 ± 3. The resulting molecular weight is thus 3692 ± 3 amu (theoretical value 3695 amu).

Example 27

Synthesis of $Arg^{26,34}$,Lys^{8}(N^{ε}-(γ-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37).

To a mixture of $Arg^{26,34}$, Lys^{8}-GLP-1 (7-37)-OH (10.3 mg, 3 μmol), EDPA (10.8 mg, 83 μmol), NMP (1.44 ml) and water (0.72 ml) was added a solution of Pal-Glu(ONSu)-OBu^{t} (4.8 mg, 8.9 μmol), prepared as described in PCT application no. PCT/DK97/00340, in NMP (120 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 70 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (4.9 mg, 65.3 μmol) in water (490 μl). A 0.5% aqueous solution of ammonium acetate (30 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (3.2 mg, 28%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3836 ± 3. The resulting molecular weight is thus 3835 ± 3 AMU (theoretical value 3836 AMU).

Example 28

Synthesis of Lau-Glu(ONSu)-OBu^{t}.

To a solution of H-Glu-OBu^{t} (3 g, 15 mmol) in DMF (344 ml) was added EDPA (2.58 ml, 15 mmol) and a solution of Lau-ONSu (4.5 g, 15 mmol), prepared in a similar manner as described for Cap-ONSu in example 11, in DMF (74 ml). The resulting mixture was stirred at ambient temperature for 18 h, and the solvent removed *in vacuo*. The oily residue was partitioned between ethyl acetate (150 ml) and 5% aqueous citric acid (250 ml). The organic phase was concentrated *in vacuo*. The residue was dissolved in DMF (40 ml) and the solution added drop by drop to a 10% aqueous citric acid solution (350 ml). The precipitated product was collected, washed with water and dried *in vacuo* for 18 h to give the intermediate free acid. To solution of the free acid intermediate in DMF (25 ml) was added N-hydroxysuccinimide (1.7 g, 14.8 mmol) and a solution of N-(3-dimethylaminopropyl)-N'-

ethylcarbodiimide (2.58 g, 13.5 mmol) in dichloromethane (52 ml). The resulting mixture was stirred at room temperature for 18 h, and the solvents removed *in vacuo*. The oily residue was partitioned between dichloromethane (80 ml) and water (80 ml). The organic phase was washed with 5% aqueous citric acid, dried ($MgSO_4$), and concentrated *in vacuo* to a solid. The solid residue was crystallised from a mixture of n-heptane (77 ml) and 2-propanol (50 ml), and finally recrystallised from n-heptane (76 ml) to give the title compound (2.96 g, 46%).

Example 29

Synthesis of Arg^{34},Lys^{26}(N^{ε}-(γ-glutamyl(N^{α}-dodecanoyl))) GLP-1 (7-37).

To a mixture of Arg^{34}-GLP-1 (7-37)-OH (20.6 mg, 6.1 μmol), EDPA (22 mg, 171 μmol), NMP (2.88 ml) and water (1.44 ml) was added a solution Lau-Glu(ONSu)-OBu^t (10.2 mg, 21.2 μmol), prepared as described in example 28, in NMP (255 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 75 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (10 mg, 134 μmol) in water (100 μl). A 0.5% aqueous solution of ammonium acetate (61 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (8.2 mg, 36%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3693 ± 3. The resulting molecular weight is 3692 ± 3 AMU (theoretical value 3693 AMU).

Example 30

Synthesis of Lau-β-Ala-ONSu.

To a solution of Lau-ONSu (4.25 g, 14.3 mmol), prepared in a similar manner to in DMF (400 ml) was added EDPA (1.84 g, 14.3 mmol) and β-alanine (1.27 g, 14.3 mmol). The resulting mixture was stirred at ambient temperature for 18 h. Water (250 ml) and DMF (50 ml) were added and the solution stirred for 1 h at room temperature. The solvents were removed *in vacuo* to give a solid. The solid residue was dissolved in DMF (50 ml) and the solution added drop by drop to a 5% aqueous solution of citric acid (200 ml). The precipitate collected, washed with water (50 ml) and dried *in vacuo* to give the title compound (3.6 g, 93%).

Example 31

Synthesis of Pal-β-Ala-ONSu.

To a solution of Pal-ONSu (4.25 g, 14.3 mmol) in DMF (400 ml) was added EDPA (1.84 g, 14.3 mmol) and β-alanine (1.27 g, 14.3 mmol). The resulting mixture was stirred at ambient temperature for 18 h. Water (250 ml) and DMF (50 ml) were added and the solution stirred for 1 h at room temperature. The solvents were removed *in vacuo* to give a solid. The solid residue was dissolved in DMF (50 ml) and the solution added drop by drop to a 5% aqueous solution of citric acid (200 ml). The precipitate collected, washed with water (50 ml) and dried *in vacuo* to give the title compound (3.6 g, 93%).

Example 32

Synthesis of Myr-GABA-ONSu.

To a solution of Myr-ONSu (4 g, 12.3 mmol) in DMF (350 ml) was added EDPA (1.58 g, 12.3 mmol) and γ-aminobutyric acid (1.26 g, 12.3 mmol). The resulting mixture was stirred at ambient temperature for 18 h. Water (50 ml) was added and the solution stirred for 1 h at room temperature. The solvents were removed *in vacuo* to give a solid. The solid residue was dissolved in DMF (75 ml) and the solution added drop by drop to a 5% aqueous solution of citric acid (250 ml). The precipitate collected, washed with water (100 ml) and dried *in vacuo* to give the free acid intermediate (3.65 g, 95%). To a solution of the free acid intermediate (3 g, 9.6 mmol), N-hydroxysuccinimide (1.65 g, 14.4 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (3.67 g, 19.1 mmol) in DMF (330 ml) was stirred for 18 h at room temperature, and the solvent removed *in vacuo* to give a solid. The solid residue was dissolved in dichloromethane (100 ml) and washed with brine (100 ml). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo* to give a solid. The solid residue was recrystallised from n-heptane (75 ml) to give the title compound (2.8 g, 71%).

Example 33

Synthesis of Pal-β-Ala-ONSu.

To a solution of Pal-ONSu (0.9 g, 2.8 mmol) in DMF (100 ml) were added N-hydroxysuccinimide (0.35 g, 3 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (0.79 g, 4.1 mmol). The resulting mixture was stirred at ambient temperature for 40h, and the solvent removed *in vacuo*. The solid residue was partitioned between water (50 ml) and

dichloromethane (50 ml). The organic phase was separated, dried ($MgSO_4$) and the solvent removed *in vacuo* to give the title compound (1.1 g, 94%).

Example 34

Synthesis of Arg^{34},Lys^{26}(N^{ε}-(β-alanyl(N^{α}-hexadecanoyl))) GLP-1 (7-37).

To a mixture of Arg^{34}-GLP-1 (7-37)-OH (19.2 mg, 5.7 μmol), EDPA (20.5 mg, 159 μmol), NMP (2.7 ml) and water (1.35 ml) was added a solution Pal-β-Ala-ONSu (7.2 mg, 17 μmol), prepared as described in example 33, in NMP (181 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (9.3 mg, 125 μmol) in water (93 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (11.6 mg, 55%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3694 ± 3. The resulting molecular weight is thus 3693 ± 3 AMU (theoretical value 3693 AMU).

Example 35

Synthesis of Pal-Glu(OBu^t)-ONSu.

To a solution of H-Glu(OH)-OBu^t (2.7 g, 11.3 mmol) and Pal-ONSu (3.98 g, 11.3 mmol) in DMF (300 ml) was added EDPA (3.2 g, 24.8 mmol). The resulting mixture was stirred at ambient temperature for 18h, and the solvent concentrated *in vacuo* to give an oil. The oily residue was dissolved in DMF (60 ml) and the solution added drop by drop to a 10% aqueous solution of citric acid (300 ml) whereby a precipitate was formed. The precipitate was collected, washed with cold water (25 ml), and dried *in vacuo* to give free acid intermediate (4.44 g, 89%). The free acid intermediate (4 g, 9.1 mmol) was dissolved in DMF (50 ml) and N-hydroxysuccinimide (1.15 g, 10 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.6 g, 13.6 mmol) were added. The resulting mixture was stirred at room temperature for 60h, the solvent concentrated *in vacuo* to give the crude title compound (8.2 g)

Example 36

Synthesis of Arg^{34},Lys^{26}(N^{ε}-(α-glutamyl(N^{α}-hexadecanoyl))) GLP-1 (7-37).

To a mixture of Arg^{34}-GLP-1 (7-37)-OH (25.6 mg, 7.6 μmol), EDPA (27.4 mg, 212 μmol), NMP (3.5 ml) and water (1.75 ml) was added a solution of Pal-Glu(OBu^t)-ONSu (12.2 mg, 22.7 μmol), prepared as described in example 35, in NMP (305 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 100 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.5 mg, 168 μmol) in water (125 μl). A 0.5% aqueous solution of ammonium acetate (72.5 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (30 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (6.1 mg, 22%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3751 ± 3. The resulting molecular weight is thus 3750 ± 3 AMU (theoretical value 3751 AMU).

Example 37

Synthesis of Ste-GABA-ONSu.

To a solution of Ste-ONSu (3 g, 7.9 mmol) in DMF (270 ml) was added EDPA (1 g, 7.9 mmol) and a solution of γ-aminobutyric acid (0.81 g, 7.9 mmol) in water (40 ml). The resulting suspension was stirred at ambient temperature for 18 h, and then concentrated *in vacuo* to a final volume of 50 ml. The resulting suspension was added to a 5% aqueous solution of citric acid (500 ml) whereby a precipitate is formed. The precipitate was collected and washed with water (50 ml), and dried *in vacuo* for 4h to give the free acid intermediate (2.8 g, 97%). To a mixture of the free acid intermediate (2.6 g, 7 mmol), N-hydroxysuccinimide (1.21 g, 10.5 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.69 g, 14 mmol) in NMP (300 ml) was stirred for 70 h, and the solvent removed *in vacuo* to give a solid. The solid residue was dissolved in dichloromethane (100 ml) and washed with brine (2x100 ml). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo* to give a solid. The solid residue was recrystallised from n-heptane (75 ml) to give the title compound (2.2 g, 67%).

Example 38

Synthesis of Pal-Isonip-ONSu.

To a suspension of 1-hexadecanoylbenzotriazole (3 g, 8.4 mmol), prepared as described in the literature (Kreutzberger; van der Goot, Arch.Pharm., 307, 1974), in DMF (350 ml) were added EDPA (1.08 g, 8.4 mmol) and a solution of piperidine-4-carboxylic acid in water (20 ml). The resulting suspension was stirred at room temperature for 12d, and then concentrated *in vacuo* to an oil. The oily residue was added drop by drop to a 5% aqueous solution of citric acid (300 ml) whereby a precipitate was formed. The precipitate was collected and washed with water (50 ml), dried *in vacuo* for 2 h to give the free acid intermediate (3 g, 97%). To a solution of the free acid intermediate (2.8 g, 7.6 mmol), N-hydroxysuccinimide (1.31 g, 11.4 mmol) in DMF (250 ml) was added N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.92 g, 15.2 mmol). The resulting mixture was stirred at ambient temperature for 18h, and the solvent removed *in vacuo* to give an oil. The oily residue was dissolved in dichloromethane (100 ml), washed with brine (50 ml), dried ($MgSO_4$) and concentrated *in vacuo* to give the crude title compound (4.1 g, quant.).

Example 39

Synthesis of Arg^{34},Lys^{26}(N^{ε}-(piperidinyl-4-carbonyl(N-hexadecanoyl))) GLP-1 (7-37).

To a mixture of Arg^{34}-GLP-1 (7-37)-OH (25 mg, 7.4 μmol), EDPA (26.7 mg, 207 μmol), NMP (3.5 ml) and water (1.75 ml) was added a solution Pal-Isonip-ONSu (13.7 mg, 30 μmol), prepared as described in example 38 in NMP (343 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 90 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (12.2 mg, 163 μmol) in water (122 μl). The reaction mixture was purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (12 mg, 44%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3734 ± 3. The resulting molecular weight is thus 3733 ± 3 AMU (theoretical value 3733 AMU).

Example 40

Synthesis of Arg^{34},Lys^{26}(N^{ε}-(γ-glutamyl(N^{α}-decanoyl))) GLP-1 (7-37).

To a mixture of Arg^{34}-GLP-1 (7-37)-OH (25 mg, 7.4 μmol), EDPA (26.7 mg, 207 μmol), NMP (3.5 ml) and water (1.75 ml) was added a solution of Cac-Glu(ONSu)-OBu^t (10 mg, 22.1 μmol) in NMP (252 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 140 min. at room temperature. The reaction was quenched by the

addition of a solution of glycine (12.2 mg, 162 μmol) in water (122 μl). A 0.5% aqueous solution of ammonium acetate (73 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised compound washed with 5% aqueous acetonitril (25 ml), and finally liberated from the cartridge by elution with TFA (25 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (12.2 mg, 45%) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 3669.7 ± 3. The resulting molecular weight is thus 3668.7 ± 3 amu (theoretical value 3667 amu).

Examples of pharmaceutical formulations

In the following "8 mM phosphate buffer of pH 7.4" is intended to mean:
4 mM NaH_2PO_4, $2H_2O$ and 4 mM Na_2HPO_4, $2H_2O$;
pH adjusted to 7.4 (using Sodium Hydroxide and/or Hydrochloric acid).

In the following "Compound 1" is intended to mean: Arg^{34},Lys^{26}(N^{ε}-($^{\gamma}$-Glu(N^{α}-tetradecanoyl))) GLP-1 (7-37).

In the following "Compound 2" is intended to mean: Arg^{34},Lys^{26}(N^{ε}-($^{\gamma}$-Glu(N^{α}-hexadecanoyl))) GLP-1 (7-37).

In the following "Compound 3" is intended to mean: $Arg^{26,34}$,Lys^{36}(N^{ε}-($^{\gamma}$-Glu(N^{α}-hexadecanoyl))) GLP-1 (7-36).

In the following "Compound 4" is intended to mean: Arg^{26},Lys^{34}(N^{ε}-($^{\gamma}$-Glu(N^{α}-hexadecanoyl))) GLP-1 (7-37).

In the following "Compound 5" is intended to mean: Gly^{8},Glu^{37},$Arg^{26,34}$,Lys^{38}(N^{ε}-($^{\gamma}$-Glu(N^{α}-hexadecanoyl))) GLP-1 (7-38).

General example A

Compound	2 - 7.5 mg/ml
Mannitol	34-50 mg/ml

Phenol 5 - 7.5 mg/ml

8 mM phosphate buffer of pH 7.4

Mannitol and phenol were dissolved in the phosphate buffer preadjusted to pH 7.4. Hereafter, the Compound were dissolved under slow stirring. The pH were adjusted to 7.4 using Sodium Hydroxide and/or Hydrochloric Acid. Finally, the formulation were sterilised by filtration through an appropriate filter.

The following specific formulations were produced using the procedure under general example A:

Example A1

Compound 1	2.0	mg/ml
Mannitol	38	mg/ml
Phenol	5	mg/ml
8 mM phosphate buffer of pH 7.4	ad 100 ml	

Example A2

Compound 1	5	mg/ml
Mannitol	36.9	mg/ml
Phenol	5	mg/ml
8 mM phosphate buffer of pH 7.4	ad 100 ml	

Example A3

Compound 1	7.5	mg/ml
Mannitol	34	mg/ml
Phenol	7.5	mg/ml
8 mM phosphate buffer of pH 7.4	ad 100 ml	

Example A4

Compound 2	2.0	mg/ml

Mannitol	38	mg/ml
Phenol	5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example A5

Compound 2	5	mg/ml
Mannitol	36.9	mg/ml
Phenol	5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example A6

Compound 2	7.5	mg/ml
Mannitol	34	mg/ml
Phenol	7.5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example A7

Compound 3	2.0	mg/ml
Mannitol	38	mg/ml
Phenol	5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example A8

Compound 3	5	mg/ml
Mannitol	36.9	mg/ml
Phenol	5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example A9

Compound 3	7.5	mg/ml
Mannitol	34	mg/ml
Phenol	7.5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example A10

Compound 4	2.0	mg/ml
Mannitol	38	mg/ml
Phenol	5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example A11

Compound 4	5	mg/ml
Mannitol	36.9	mg/ml
Phenol	5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example A12

Compound 4	7.5	mg/ml
Mannitol	34	mg/ml
Phenol	7.5	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

General example B

Compound	2 - 7.5 mg/ml
Mannitol	19-25 mg/ml
Benzyl Alcohol	14-18 mg/ml
8 mM phosphate	buffer of pH 7.4

Mannitol and benzyl alcohol were dissolved in the phosphate buffer preadjusted to pH 7.4. Hereafter, the Compound were dissolved under slow stirring. The pH were adjusted to 7.4 using Sodium Hydroxide and/or Hydrochloric Acid. Finally, the formulation were sterilised by filtration through an appropriate filter.

The following specific formulations were produced using the procedure under general example B:

Example B1

Compound 1	2.0	mg/ml
Mannitol	25	mg/ml
Benzyl alcohol	14	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example B2

Compound 1	7.5	mg/ml
Mannitol	19	mg/ml
Benzyl alcohol	18	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example B3

Compound 2	2.0	mg/ml
Mannitol	25	mg/ml
Benzyl alcohol	14	mg/ml
8 mM phosphate		
buffer of pH 7.4	ad 100 ml	

Example B4

Compound 2	7.5	mg/ml
Mannitol	19	mg/ml
Benzyl alcohol	18	mg/ml
8 mM phosphate		

buffer of pH 7.4 ad 100 ml

Example B5

Compound 3	2.0	mg/ml
Mannitol	25	mg/ml
Benzyl alcohol	14	mg/ml

8 mM phosphate
buffer of pH 7.4 ad 100 ml

Example B6

Compound 3	7.5	mg/ml
Mannitol	19	mg/ml
Benzyl alcohol	18	mg/ml

8 mM phosphate
buffer of pH 7.4 ad 100 ml

Example B7

Compound 5	2.0	mg/ml
Mannitol	25	mg/ml
Benzyl alcohol	14	mg/ml

8 mM phosphate
buffer of pH 7.4 ad 100 ml

Example B8

Compound 5	7.5	mg/ml
Mannitol	19	mg/ml
Benzyl alcohol	18	mg/ml

8 mM phosphate
buffer of pH 7.4 ad 100 ml

General example C

Compound	2 - 7.5	mg/ml
Mannitol	42-44	mg/ml
Metacresol	2.5-4.0	mg/ml

8 mM phosphate buffer of pH 7.4

Mannitol and metacresol were dissolved in the phosphate buffer preadjusted to pH 7.4. Hereafter, the Compound were dissolved under slow stirring. The pH were adjusted to 7.4 using Sodium Hydroxide and/or Hydrochloric Acid. Finally, the formulation were sterilised by filtration through an appropriate filter.

The following specific formulations were produced using the procedure under general example C:

Example C1

Compound 1	2.0	mg/ml
Mannitol	44	mg/ml
Metacresol	2.5	mg/ml
8 mM phosphate buffer of pH 7.4	ad 100 ml	

Example C2

Compound 1	7.5	mg/ml
Mannitol	42	mg/ml
Metacresol	4	mg/ml
8 mM phosphate buffer of pH 7.4	ad 100 ml	

Example C3

Compound 2	2.0	mg/ml
Mannitol	44	mg/ml
Metacresol	2.5	mg/ml
8 mM phosphate buffer of pH 7.4	ad 100 ml	

Example C4

Compound 2	7.5	mg/ml
Mannitol	42	mg/ml

Metacresol	4	mg/ml
8 mM phosphate		
buffer of pH 7.4 ad 100 ml		

Example C5

Compound 3	2.0	mg/ml
Mannitol	44	mg/ml
Metacresol	2.5	mg/ml
8 mM phosphate		
buffer of pH 7.4 ad 100 ml		

Example C6

Compound 3	7.5	mg/ml
Mannitol	42	mg/ml
Metacresol	4	mg/ml
8 mM phosphate		
buffer of pH 7.4 ad 100 ml		

Example C7

Compound 5	2.0	mg/ml
Mannitol	44	mg/ml
Metacresol	2.5	mg/ml
8 mM phosphate		
buffer of pH 7.4 ad 100 ml		

Example C8

Compound 5	7.5	mg/ml
Mannitol	42	mg/ml
Metacresol	4	mg/ml
8 mM phosphate		
buffer of pH 7.4 ad 100 ml		

CLAIMS

1. A pharmaceutical composition comprising a GLP-1 derivative which has a helix content as measured by CD at 222 nm in H_2O at 22 ± 2 °C exceeding 25%, preferably in the range of 25% to 50%, at a peptide concentration of about 10 μM .

2. A pharmaceutical composition according to claim 1, wherein the concentration of GLP-1 derivative is not less than 0.5 mg/ml, preferably not less than about 5 mg/ml, more preferred not less than about 10 mg/ml and, preferably, not more than about 100 mg/ml.

3. A pharmaceutical composition according to claim 1 or 2, comprising a GLP-1 derivative wherein at least one amino acid residue of the parent peptide has a lipophilic substituent attached.

4. A pharmaceutical composition according to claim 3, comprising a GLP-1 derivative having a lipophilic substituent which is attached to any one of the amino acid residues in position 18-38, preferably 26-34.

5. A pharmaceutical composition according to any one of the preceding claims, further comprising a pharmaceutically acceptable vehicle or carrier.

6. A pharmaceutical composition according to any one of the preceding claims, further comprising an isotonic agent, preferably selected from the group consisting of sodium chloride, mannitol and glycerol.

7. A pharmaceutical composition according to any one of the preceding claims, further comprising a preservative, preferably selected from the group consisting of phenol, m-cresol, methyl p-hydroxybenzoate, butyl p-hydroxybenzoate and benzyl alcohol.

8. A pharmaceutical composition according to any one of the preceding claims, further comprising a buffer, preferably selected from the group consisting of sodium acetate, citrate, glycylglycine, histidine, 2-phenylethanol and sodium phosphate.

9. A pharmaceutical composition according to any one of the preceding claims, further comprising a surfactant capable of improving the solubility and/or the stability of the GLP-1 derivative, preferable selected from poloxymer 188, tween 20 and tween 80.

10. A pharmaceutical composition according to according to any one of the preceding claims, comprising a GLP-1 derivative wherein the lipophilic substituent comprises from 4 to 40 carbon atoms, preferably from 8 to 25 carbon atoms.

11. A pharmaceutical composition according to according to any one of the preceding claims, comprising a GLP-1 derivative wherein a lipophilic substituent is attached to an amino acid residue in such a way that a carboxyl group of the lipophilic substituent forms an amide bond with an amino group of the amino acid residue.

12. A pharmaceutical composition according to any one of the claims 1-11, comprising a GLP-1 derivative wherein a lipophilic substituent is attached to an amino acid residue in such a way that an amino group of the lipophilic substituent forms an amide bond with a carboxyl group of the amino acid residue.

13. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-1 derivative wherein the lipophilic substituent is attached to the parent peptide by means of a spacer.

14. A pharmaceutical composition according to claim 13, wherein the spacer is an unbranched alkane α,ω-dicarboxylic acid group having from 1 to 7 methylene groups, preferably two methylene groups, which form a bridge between an amino group of the parent peptide and an amino group of the lipophilic substituent.

15. A pharmaceutical composition according to claim 13, wherein the spacer is an amino acid residue except Cys, or a dipeptide such as Gly-Lys or any unbranched alkane α,ω-aminoacid having from 1 to 7 methylene groups, preferably 2-4 methylene groups, which form a bridge between an amino group of the parent peptide and an amino group of the lipophilic substituent.

16. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-1 derivative wherein the lipophilic substituent comprises a partially or completely hydrogenated cyclopentanophenathrene skeleton.

17. A pharmaceutical composition according to any one of claims 3 to 15, wherein the lipophilic substituent is a straight-chain or branched alkyl group.

18. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative wherein the lipophilic substituent is the acyl group of a straight-chain or branched fatty acid.

19. A pharmaceutical composition according to claim 18 wherein the acyl group is selected from the group comprising $CH_3(CH_2)_nCO$-, wherein n is 4 to 38, preferably $CH_3(CH_2)_6CO$-, $CH_3(CH_2)_8CO$-, $CH_3(CH_2)_{10}CO$-, $CH_3(CH_2)_{12}CO$-, $CH_3(CH_2)_{14}CO$-, $CH_3(CH_2)_{16}CO$-, $CH_3(CH_2)_{18}CO$-, $CH_3(CH_2)_{20}CO$- and $CH_3(CH_2)_{22}CO$-.

20. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative wherein the lipophilic substituent is an acyl group of a straight-chain or branched alkane α,ω-dicarboxylic acid.

21. A pharmaceutical composition according to claim 20 wherein the acyl group is selected from the group comprising $HOOC(CH_2)_mCO$-, wherein m is from 4 to 38, preferably from 4 to 24, more preferred selected from the group comprising $HOOC(CH_2)_{14}CO$-, $HOOC(CH_2)_{16}CO$-, $HOOC(CH_2)_{18}CO$-, $HOOC(CH_2)_{20}CO$- and $HOOC(CH_2)_{22}CO$-.

22. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative wherein the lipophilic substituent is a group of the formula $CH_3(CH_2)_p((CH_2)_qCOOH)CHNH\text{-}CO(CH_2)_2CO$-, wherein p and q are integers and p+q is an integer of from 8 to 33, preferably from 12 to 28.

23. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative, wherein the lipophilic substituent is a group of the formula $CH_3(CH_2)_rCO\text{-}NHCH(COOH)(CH_2)_2CO$-, wherein r is an integer of from 10 to 24.

24. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative, wherein the lipophilic substituent is a group of the formula $CH_3(CH_2)_sCO\text{-}NHCH((CH_2)_2COOH)CO$-, wherein s is an integer of from 8 to 24.

25. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative, wherein the lipophilic substituent is a group of the formula $\text{-}NHCH(COOH)(CH_2)_4NH\text{-}CO(CH_2)_uCH_3$, wherein u is an integer of from 8 to 18.

26. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative, wherein the lipophilic substituent is a group of the formula

-$NHCH(COOH)(CH_2)_4NH$-$COCH((CH_2)_2COOH)NH$-$CO(CH_2)_wCH_3$, wherein w is an integer of from 10 to 16.

27. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative, wherein the lipophilic substituent is a group of the formula -$NHCH(COOH)(CH_2)_4NH$-$CO(CH_2)_2CH(COOH)NH$-$CO(CH_2)_xCH_3$, wherein x is an integer of from 10 to 16.

28. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-1 derivative wherein the lipophilic substituent is a group of the formula -$NHCH(COOH)(CH_2)_4NH$-$CO(CH_2)_2CH(COOH)NH$-$CO(CH_2)_yCH_3$, wherein y is zero or an integer of from 1 to 22.

29. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-1 derivative wherein the parent peptide is GLP-1(A-B) wherein A is an integer from 1 to 7 and B is an integer from 38 to 45, or an analogue thereof.

30. A pharmaceutical composition according to claim 29, wherein the parent peptide is selected from the group comprising GLP-1(7-35); GLP-1(7-36); GLP-1(7-36)amide; GLP-1(7-37); GLP-1(7-38); GLP-1(7-39); GLP-1(7-40) and GLP-1(7-41); and analogues thereof.

31. A pharmaceutical composition according to claim 29, wherein the parent peptide is selected from the group comprising GLP-1(1-35); GLP-1(1-36); GLP-1(1-36)amide; GLP-1(1-37); GLP-1(1-38); GLP-1(1-39); GLP-1(1-40); GLP-1(1-41); and an analogues thereof.

32. A pharmaceutical composition according to any of the preceding claims comprising a GLP-1 derivative wherein a total of up to fifteen, preferably up to ten, more preferably up to six, amino acid residues have been exchanged with any α-amino acid residue which can be coded for by the genetic code.

33. A pharmaceutical composition according to any of the preceding claims, comprising a GLP-1 derivative wherein the parent peptide is selected from the group comprising Arg^{26}-GLP-1(7-37); Arg^{34}-GLP-1(7-37); Lys^{36}-GLP-1(7-37); $Arg^{26,34}Lys^{36}$-GLP-1(7-37); $Arg^{26,34}Lys^{38}$GLP-1(7-38); $Arg^{26,34}Lys^{39}$-GLP-1(7-39); $Arg^{26,34}Lys^{40}$-GLP-1(7-40); $Arg^{26}Lys^{36}$-GLP-1(7-37); $Arg^{34}Lys^{36}$-GLP-1(7-37); $Arg^{26}Lys^{39}$-GLP-1(7-39); $Arg^{34}Lys^{40}$-GLP-1(7-40); $Arg^{26,34}Lys^{36,39}$-GLP-1(7-39); $Arg^{26,34}Lys^{36,40}$-GLP-1(7-40); Gly^8Arg26-GLP-1(7-37); Gly^8Arg^{34}-GLP-1(7-37); Gly^8Lys^{36}-GLP-1(7-37); $Gly^8Arg^{26,34}Lys^{36}$-GLP-1(7-37);

$Gly^8Arg^{26,34}Lys^{39}$-GLP-1(7-39); $Gly^8Arg^{26,34}Lys^{40}$-GLP-1(7-40); $Gly^8Arg^{26}Lys^{36}$-GLP-1(7-37); $Gly^8Arg^{34}Lys^{36}$-GLP-1(7-37); $Gly^8Arg^{26}Lys^{39}$-GLP-1(7-39); $Gly^8Arg^{34}Lys^{40}$-GLP-1(7-40); $Gly^8Arg^{26,34}Lys^{36,39}$-GLP-1(7-39) and $Gly^8Arg^{26,34}Lys^{36,40}$-GLP-1(7-40).

34. A pharmaceutical composition according to any of the claims 1 to 32, comprising a GLP-1 derivative, wherein the parent peptide is selected from the group comprising $Arg^{26,34}Lys^{38}$GLP-1(7-38); $Arg^{26,34}Lys^{39}$GLP-1(7-39); $Arg^{26,34}Lys^{40}$GLP-1(7-40); $Arg^{26,34}Lys^{41}$GLP-1(7-41); $Arg^{26,34}Lys^{42}$GLP-1(7-42); $Arg^{26,34}Lys^{43}$GLP-1(7-43); $Arg^{26,34}Lys^{44}$GLP-1(7-44); $Arg^{26,34}Lys^{45}$GLP-1(7-45); $Arg^{26,34}Lys^{38}$GLP-1(1-38); $Arg^{26,34}Lys^{39}$GLP-1(1-39); $Arg^{26,34}Lys^{40}$GLP-1(1-40); $Arg^{26,34}Lys^{41}$GLP-1(1-41); $Arg^{26,34}Lys^{42}$GLP-1(1-42); $Arg^{26,34}Lys^{43}$GLP-1(1-43); $Arg^{26,34}Lys^{44}$GLP-1(1-44); $Arg^{26,34}Lys^{45}$GLP-1(1-45); $Arg^{26,34}Lys^{38}$GLP-1(2-38); $Arg^{26,34}Lys^{39}$GLP-1(2-39); $Arg^{26,34}Lys^{40}$GLP-1(2-40); $Arg^{26,34}Lys^{41}$GLP-1(2-41); $Arg^{26,34}Lys^{42}$GLP-1(2-42); $Arg^{26,34}Lys^{43}$GLP-1(2-43); $Arg^{26,34}Lys^{44}$GLP-1(2-44); $Arg^{26,34}Lys^{45}$GLP-1(2-45); $Arg^{26,34}Lys^{38}$GLP-1(3-38); $Arg^{26,34}Lys^{39}$GLP-1(3-39); $Arg^{26,34}Lys^{40}$GLP-1(3-40); $Arg^{26,34}Lys^{41}$GLP-1(3-41); $Arg^{26,34}Lys^{42}$GLP-1(3-42); $Arg^{26,34}Lys^{43}$GLP-1(3-43); $Arg^{26,34}Lys^{44}$GLP-1(3-44); $Arg^{26,34}Lys^{45}$GLP-1(3-45); $Arg^{26,34}Lys^{38}$GLP-1(4-38); $Arg^{26,34}Lys^{39}$GLP-1(4-39); $Arg^{26,34}Lys^{40}$GLP-1(4-40); $Arg^{26,34}Lys^{41}$GLP-1(4-41); $Arg^{26,34}Lys^{42}$GLP-1(4-42); $Arg^{26,34}Lys^{43}$GLP-1(4-43); $Arg^{26,34}Lys^{44}$GLP-1(4-44); $Arg^{26,34}Lys^{45}$GLP-1(4-45); $Arg^{26,34}Lys^{38}$GLP-1(5-38); $Arg^{26,34}Lys^{39}$GLP-1(5-39); $Arg^{26,34}Lys^{40}$GLP-1(5-40); $Arg^{26,34}Lys^{41}$GLP-1(5-41); $Arg^{26,34}Lys^{42}$GLP-1(5-42); $Arg^{26,34}Lys^{43}$GLP-1(5-43); $Arg^{26,34}Lys^{44}$GLP-1(5-44); $Arg^{26,34}Lys^{45}$GLP-1(5-45); $Arg^{26,34}Lys^{38}$GLP-1(6-38); $Arg^{26,34}Lys^{39}$GLP-1(6-39); $Arg^{26,34}Lys^{40}$GLP-1(6-40); $Arg^{26,34}Lys^{41}$GLP-1(6-41); $Arg^{26,34}Lys^{42}$GLP-1(6-42); $Arg^{26,34}Lys^{43}$GLP-1(6-43); $Arg^{26,34}Lys^{44}$GLP-1(6-44); $Arg^{26,34}Lys^{45}$GLP-1(6-45); $Arg^{26}Lys^{38}$GLP-1(1-38); $Arg^{34}Lys^{38}$GLP-1(1-38); $Arg^{26,34}Lys^{36,38}$GLP-1(1-38); $Arg^{26}Lys^{38}$GLP-1(7-38); $Arg^{34}Lys^{38}$GLP-1(7-38); $Arg^{26,34}Lys^{36,38}$GLP-1(7-38); $Arg^{26,34}Lys^{38}$GLP-1(7-38); $Arg^{26}Lys^{39}$GLP-1(1-39); $Arg^{34}Lys^{39}$GLP-1(1-39); $Arg^{26,34}Lys^{36,39}$GLP-1(1-39); $Arg^{26}Lys^{39}$GLP-1(7-39); $Arg^{34}Lys^{39}$GLP-1(7-39) and $Arg^{26,34}Lys^{36,39}$GLP-1(7-39).

35. A method for improving the solubility and/or stability of GLP-1 or a fragment or an analogue thereof, characterised in that a lipophilic substituent is introduced on any one of the amino acid residues of the parent peptide.

36. A method according to claim 35, wherein a lipophilic substituent is introduced on any one of the amino acid residues in position 18-38, preferably 26-34.

37. A method according to claim 35 or 36, wherein the lipophilic substituent comprises from 4 to 40 carbon atoms, preferably from 8 to 25 carbon atoms.

38. A method according to any one of claim 35 to 37, wherein the lipophilic substituent is the acyl group of a straight-chain or branched fatty acid.

39. A method according to any one of claim 36, wherein the acyl group is selected from the group comprising $CH_3(CH_2)_nCO$-, wherein n is 4 to 38, preferably $CH_3(CH_2)_6CO$-, $CH_3(CH_2)_8CO$-, $CH_3(CH_2)_{10}CO$-, $CH_3(CH_2)_{12}CO$-, $CH_3(CH_2)_{14}CO$-, $CH_3(CH_2)_{16}CO$-, $CH_3(CH_2)_{18}CO$-, $CH_3(CH_2)_{20}CO$- and $CH_3(CH_2)_{22}CO$-.

40. A method according to any one of claims 35 to 39, wherein the GLP-1 is GLP-1(A-B) wherein A is an integer from 1 to 7 and B is an integer from 38 to 45, or an analogue thereof.

41. A method according to claim 40, wherein the GLP-1 is selected from the group comprising GLP-1(7-35); GLP-1(7-36); GLP-1(7-36)amide; GLP-1(7-37); GLP-1(7-38); GLP-1(7-39); GLP-1(7-40) and GLP-1(7-41); and analogues thereof.

42. A method according to claim 40, wherein the GLP-1 is selected from the group comprising GLP-1(1-35); GLP-1(1-36); GLP-1(1-36)amide; GLP-1(1-37); GLP-1(1-38); GLP-1(1-39); GLP-1(1-40); GLP-1(1-41); and an analogues thereof.

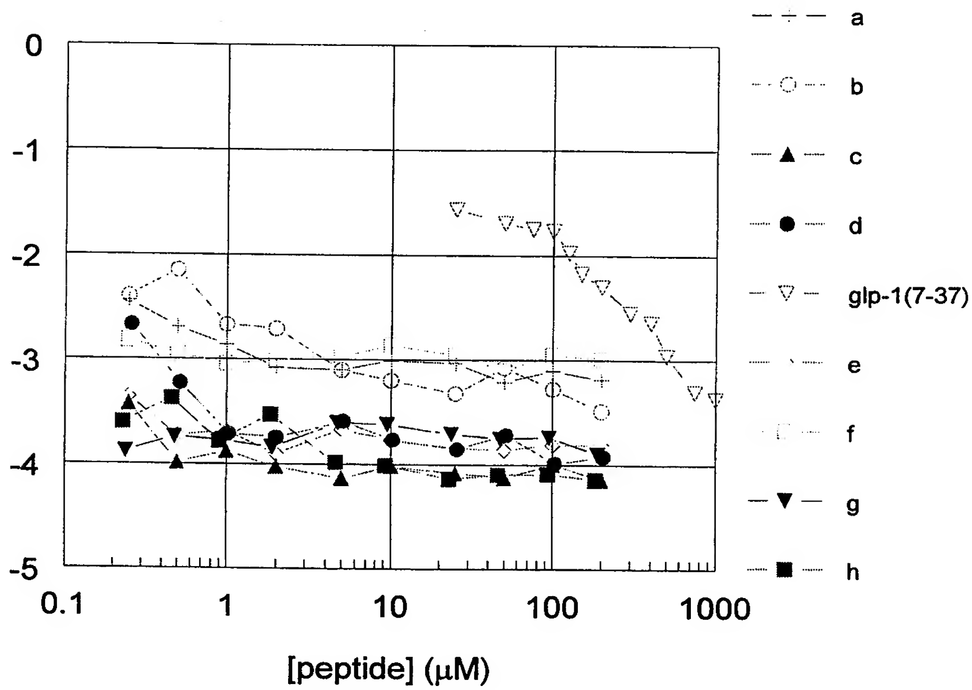

Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00084

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/26, C07K 14/605
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9808871 A1 (NOVO NORDISK A/S), 5 March 1998 (05.03.98) --	1-42
X	Journal of Pharmaceutical Sciences, Volume 89, No 8, August 1994, Yesook Kim et al, "FT-IR and Near-Infared FT-Raman Studies of the Secondary Structure of Insulinotropin in the Solid State; alpha-Helix to Beta-Sheet Concersion Induced by Phenol and/or by High Shear Force" page 1173 - page 1180 --	1-42

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 1999	20 -06- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Hampus Rystedt/EÖ Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00084

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Pharmaceutical Research, Volume 15, No 2, 1998, Dean K. Clodfelter et al, "Effects of Non-Covalent Self-Association on the Subcutaneous Absorption of a Therapeutic Peptide", page 254 - page 262, see especially abstract, figure 1, table 1	1-34
A		35-42
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A	US 5614492 A (JOEL F. HABENER), 25 March 1997 (25.03.97), abstract, claims	1-42
	--	
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